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EXPERIMENTAL INFECTION OF CHICK EMBRYOS WITH ENTAMOEBA INVADENS[1]

BY EUGENE MEEROVITCH

Abstract

Entamoeba invadens in chick embryos, incubated at 30° C., invaded and produced changes in the liver. The embryos did not survive more than six days at 30° C. after inoculation of the fluid medium, with or without *E. invadens*, and the inoculated amoebae did not survive the death of the embryo.

In a previous publication (2) the author reported the preliminary results of infecting developing chick embryos with bacteria-free *Entamoeba invadens*. This parasite, which closely resembles *Entamoeba histolytica* morphologically, had been reported naturally infecting certain snakes and lizards, and some other species of these two classes of reptiles were also experimentally infected with it (1). In these hosts *E. invadens* invades the tissues of the large intestine, gaining early access to the blood stream by which it is carried to the liver and later to other organs where it sets up new foci of infection, usually with fatal results for the host. The disease in snakes and the pathological conditions produced in them are described by Ratcliffe and Geiman (4).

Two strains of *E. invadens* (I.P. and P.Z. strains), which are maintained in bacteria-free cultures in this laboratory (3), were used in these experiments. The methods of inoculation and incubation of the embryos and of examination of their livers for the presence of amoebae were the same as those described in the first paper (2).

Results

Cultures of the two strains of bacteria-free *E. invadens* were inoculated into a chorioallantoic membranal blood vessel of 79 white Leghorn embryos, ranging in age from 9 to 15 days. Each embryo received 0.1 ml. of fluid

[1]*Manuscript received in original form June 1, 1955, and as revised, October 31, 1955. Contribution from the Institute of Parasitology, McGill University, Macdonald College P.O., Quebec, Canada, with financial assistance from the National Research Council of Canada and the Banting Research Foundation.*

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containing approximately 5000 amoebae, although no attempt was made to determine the number of organisms accurately in every case. The cultures used had been incubated at 20°–25° C.

The embryos were then incubated for from one to seven days at 30° C. They were then removed from the egg and their livers examined for the presence of amoebae either by direct microscopical examination, by placing liver fragments in a suitable culture medium for the amoebae, or by examining stained sections of the liver.

On gross examination most of the livers that were found to be invaded by amoebae were red and patchy in appearance, but this condition was confined to the left lobe in all cases. Histological sections revealed blocked capillaries and sinusoids and a general haemorrhagic condition of the organ.

Seven embryos (19.4% of the number found infected) had abscesses in the livers. These abscesses were whitish-yellow in color and were confined to the tip of the left lobe. Vegetative amoebae were found in greater numbers in the tissues immediately surrounding the abscesses than in other portions of the liver. In general, amoebae were never found in large numbers, so that extremely careful and prolonged examination of the liver sections had to be made in order to find any. Only in a few cases were positive diagnoses based on the culture method. Amoebic cysts were never found in the tissues of the embryos.

TABLE I

NUMBER OF EMBRYOS ALIVE AND DEAD AT EXAMINATION, SHOWING PRESENCE OR ABSENCE OF *E. invadens* (I.P. AND P.Z. STRAINS) IN THE LIVERS

		Number of embryos alive	Number of embryos dead	Total
Amoebae present in liver	I.P. strain	12	8	20
	P.Z. strain	15	1	16
Amoebae not present in liver	I.P. strain	8	13	21
	P.Z. strain	12	10	22
Total		47	32	79

TABLE II

EMBRYOS' SURVIVAL RATE, PRESENCE OF *E. invadens*, AND LIVER PATHOLOGY

	Days of incubation at 30° C.						
Embryos	1	2	3	4	5	6	7
(*a*) Number examined	3	6	24	22	10	12	2
(*b*) Per cent alive	100	83	75	77	40	0	0
(*c*) Per cent positive	67	62	54	45	50	8	0
(*d*) Per cent with haemorrhagic livers	67	83	75	59	60	67	100

TABLE III

LACK OF RELATIONSHIP BETWEEN THE AGE OF CULTURES OF *E. invadens* AND THEIR INFECTIVITY TO CHICK EMBRYOS

Embryos	Age of culture in days						
	Up to 10	10–20	20–30	30–40	40–50	50–60	60–70
Number inoculated	9	7	9	21	16	9	8
Number positive	4	3	6	9	9	1	4
Approximate percentage positive	44	43	67	43	56	11	50

The results obtained may be summarized as follows:

1. Of the total number of 79 embryos, 36 (or 45.5%) had amoebae in the livers; of the 41 embryos inoculated with the I.P. strain of *E. invadens*, 20 (or 48.8%) were positive, and of the 38 inoculated with the P.Z. strain, 16 (or 42.1%) were positive (Table I).

2. Forty-seven embryos were alive when examined and 32 were dead. Twenty of the living embryos had been inoculated with the I.P strain and 27 with the P.Z. strain (Table I).

3. As the period of incubation at 30° C. was increased from one to seven days, there was a corresponding decrease in the percentage of embryos remaining alive on successive days (Table II (*b*)).

4. The percentage of embryos with amoebae in their livers was greater in those examined during the first few days of incubation, fell sharply on the sixth day, and became zero on the seventh (Table II (*c*)).

5. The percentage of embryos whose livers presented a pathological appearance corresponded roughly to the percentage of embryos with amoebae in their livers during the first five days, but was much higher on the sixth day and reached 100% on the seventh day (Table II (*d*)).

6. The age of the culture used for inoculation did not affect the establishment of an infection in the embryo (Table III).

7. No significant differences between the two strains of *E. invadens* in establishing infection in chick embryos were observed in these experiments.

The following control experiments were performed:

Two 14-day-old and twelve 12-day-old embryos were inoculated intravenously with sterile saline–serum medium (the same medium in which the amoebae were inoculated into the embryos). The first two controls received 0.05 ml. each and the other twelve 0.1 ml. of the medium. After inoculation they were incubated at 30° C. and candled daily to determine whether they were alive or dead.

Groups of two embryos were removed from the egg on the second, third, fourth, and fifth day of incubation. All these were alive when examined. Of the remaining six controls in this group, four died on the sixth day, and two on the seventh day. The livers of all 14 embryos were normal.

In the second group of controls, consisting of six 12-day-old embryos, the shells were drilled and windows in them were made, as for inoculation, but no inoculation was performed. Following the operation they were incubated at 30° C. One died on the fifth day, two on the sixth day, two on the seventh, and the remaining one was alive when removed from the egg on the sixth day.

Six other 12-day-old embryos not treated in any way were incubated at 30° C. Two died on the sixth day and three on the seventh day; the remaining one was still alive when it was removed from the egg on the ninth day. The intact controls showed a tendency to survive slightly longer than those inoculated or drilled, but most were dead by the seventh day.

Both experimental and control embryos showed that they did not survive for more than six days at 30° C. after receiving an intravenous injection of the medium with amoebae, the medium alone, or after having parts of their shells removed as for an injection. It would seem therefore that the relatively low temperature of incubation (9° C. below normal) aided by the operative procedures, rather than the amoebae, were mainly responsible for the early death of the embryo.

Discussion

As the amoebae were inoculated intravenously into the embryos, they were carried by the blood stream to the liver, where they set up foci of infection. The pathological condition of the liver arose after the blockage of capillaries and the formation of thrombi, following the invasion of the capillary walls by the parasites.

It is suggested that the death of the embryos after six days at 30° C. caused a change in the environmental conditions for the amoebae, and that the post-mortem changes in the embryo, together with the unfavorably high temperature of incubation for the amoebae (30° C.), could be responsible for the disappearance of the latter from the livers. The changes produced by the parasites in the livers were neither altered nor magnified after the death of the embryos, because of the absence of bacteria or other microorganisms.

This sequence of events can be followed in Table II.

The presence of amoebae in the livers was shown directly by the demonstration of the parasites, and indirectly by the pathological changes produced by them. The reason why amoebae were seen in a decreasing percentage of embryos as the period of incubation at 30° C. was increased, was probably because the amoebae died and became autolyzed when the embryos died.

The death of the embryos was not caused by the infection, but by the unfavorable conditions brought about by incubation at 30° C.; the trauma received after the removal of a part of the shell and the intravenous injection of a foreign fluid probably was a contributing factor.

Amoebae were demonstrated in slightly less than half of the total number of embryos which were inoculated. As, apparently, the amoebae disappeared very soon after the death of the embryos, it does not mean that the remaining

half of the embryos never became infected. Since these numbers comprise all the embryos, alive and dead, which were examined during the seven days, those whose livers were haemorrhagic, but in which no amoebae were demonstrated, were most probably infected originally.

These experiments did not suggest that *E. invadens* multiplied readily within the tissues of the embryos. If mass multiplication had occurred, the amoebae would have been much more easily demonstrable in liver sections. The choice of 30° C. as the incubation temperature for inoculated embryos was a compromise between the optimum temperature requirements of the embryos and the amoebae and was not the optimum temperature for either the host or the parasite. The author's experience with *in vitro* cultivation of *E. invadens* has shown that only some of the amoebae subcultured from 20°–25° C. to 30° C. are able to survive and that these multiply at a slower rate than at room temperatures. However, it is possible to adapt them to grow at higher temperatures by a process of selection of the surviving organisms, and the reason why some of the embryos did not become infected at all is probably because the amoebae injected into them were unable to adapt themselves to 30° C., and so were not able to become established at this temperature. In those embryos in which amoebae were demonstrated, it is probable that only a few of the organisms introduced may have been able to survive and produce pathological changes and it is not possible to say whether this portion could multiply or merely survive.

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SPECIFICITY OF BACTERIAL VIRUS FOR XANTHOMONAS TRIFOLII[1]

By Norman James and E. B. Roslycky

Abstract

A virus that lysed its homologous culture was obtained in 20 out of 29 attempts by inoculating wheat with different cultures of *Xanthomonas trifolii*. Each virus lysed, in addition, certain but in no case all other cultures. A composite virus-filtrate lysed all 29 cultures and 157 of 191 additional isolates obtained from various kinds of cultivated seeds. It did not lyse any of the 18 cultures of *X. translucens* nor any of 17 other recognized species or subspecies.

Introduction

In previous reports from this laboratory (1, 3) it was shown that wheat seeds harbored large numbers of yellow chromogenic bacteria and that these bacteria constituted practically the total population at the dilution selected for counting on plates prepared from several samples. Similar bacteria were found on barley, oats, soybeans, and peas. A later report (2) dealt with a detailed study of these bacteria and a consideration of their taxonomic position. The opinion was expressed that they represented the same species as that studied by earlier investigators and referred to variously as *Bacterium herbicola aureum* Düggeli, *Pseudomonas trifolii* Huss, and *Flavobacterium herbicola* (Düggeli) Mack. Evidence was presented that established the validity of *Pseudomonas trifolii* Huss, and justified transferring the species to the genus *Xanthomonas*. Accordingly, the name *Xanthomonas trifolii* was proposed.

This report deals with the isolation of bacterial viruses for *Xanthomonas trifolii* and their use for the rapid identification of the species.

Materials and Methods

Freshly isolated cultures of *X. trifolii* from stored wheat were used. The procedure for obtaining the virus was based on earlier studies. Thomas (7) used oat grain extract for developing phages (viruses) specific for certain plant pathogens. Katznelson and Sutton (4) used a mixture of soil, compost, and sewage as the source of viruses for detecting internal infection of bean seeds with *X. phaseoli* and *Pseudomonas phaseolicola*, and later (5, 6) used ground cereal seed and a mixture of ground seed, composted soil, and raw sewage as the sources of viruses for the various *formae speciales* of *X. translucens*. The viruses isolated in the present study were obtained from the stored wheat from which the cultures of *X. trifolii* had been obtained at the beginning of the investigation. In each case one isolate of *X. trifolii* in 75 ml. 0.1% dextrose

[1]*Manuscript received September 26, 1955.*
Contribution from the Department of Microbiology, The University of Manitoba, Winnipeg, Manitoba, with financial assistance from the National Research Council of Canada.

nutrient broth to which was added 25 gm. ground wheat was cultured at 25° C. for 24 hr. Growth was slightly better in this medium than in nutrient broth. After incubation, coarse particles were removed by passage of the suspension through filter paper, and a bacteria–free filtrate obtained by using a sterile Seitz filter. One milliliter of filtrate and 1 ml. homologous culture were incubated in 8 ml. broth at 25° C. for 24 hr. and filtered. In most cases after two to four enrichments 0.1 ml. filtrate lysed 1 ml. of culture. At this stage each filtrate was plated and a single plaque selected: and the enrichment process was repeated through two to six serial transfers, or until 0.1 ml. filtrate lysed 1 ml. of culture. The virus-filtrate was then stored in a screw-top glass container at 4° C. for use later. In addition, duplicates of certain filtrates were stored in a frozen state.

Each virus-filtrate was tested against each culture by adding 0.1 ml. filtrate and 1 ml. of a 24 hr. culture to 8 ml. of 0.1% dextrose nutrient broth, incubating, and comparing growth with a control to which was added the culture only. A composite was prepared by mixing equal portions of selected virus-filtrates. It was tested against each of the original cultures and 191 additional cultures from stored wheat, barley, oats, flax, trefoil, brome grass, wild oats, sweet clover, *Poa*, and milk. It was tested also against 18 cultures of *X. translucens*, one culture of *X. carotae*, one of *X. campestris*, and 15 miscellaneous cultures commonly used for laboratory instruction.

Results

Lysis by Single-culture Virus-filtrates

Twenty virus-filtrates were obtained in 29 attempts with different cultures of *X. trifolii*. Nine cultures failed to produce filtrates that lysed homologous cultures completely, even at the fourth serial enrichment. Because of the close resemblance of *X. trifolii* to the pathogen, *X. translucens*, each of the 20 cultures of *X. trifolii* was tested for pathogenicity on host plants known to be susceptible to *X. translucens*. None produced disease, whereas, when tested under the same conditions, each of 18 cultures of *X. translucens*, including five pathogenic on wheat, five on barley, five on rye, and three on *Agropyron* sp. produced disease on its susceptible host.

The virus-filtrate obtained from one culture was culture specific. It lysed its homologous culture, but none of the other 28 cultures. Each of the other virus-filtrates lysed its homologous culture and variously from 1 to 19 other cultures. Each of the 20 virus-filtrates failed to lyse any of the 18 cultures of *X. translucens* referred to above. A reciprocal relationship between culture and virus-filtrate was apparent in certain cases but not in others. Culture 5 was lysed by virus-filtrates V and VIII only and virus-filtrate V lysed cultures 5 and 8 only, whereas culture 8 was lysed by virus-filtrates V and VIII and five others, and virus-filtrate VIII lysed cultures 5 and 8 and 17 other cultures. Certain pairs of cultures were lysed by the same virus-filtrates which, as a

group, differed from the groups lysing other pairs of cultures. However, certain virus-filtrates appeared in more than one group. For example, cultures 2 and 14 were lysed by virus-filtrates II, IV, VIII, XIII, XIV, XVI, XVII, and XX, and cultures 11 and 20 were lysed by II, IV, VIII, XI, XIII, XVI, XVII, and XX. The details are presented in Table I.

TABLE I

LYSIS BY SINGLE-CULTURE VIRUS-FILTRATES

Culture	Virus-filtrate																		
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
1	+						+							+					+	
2		+		+				+					+	+		+	+			+
3		+	+	+				+					+							
4		+		+									+				+			+
5					+			+												
6		+				+	+	+			+			+			+		+	+
7	+						+												+	
8		+	+	+	+			+				+	+							
9		+		+					+				+							
10		+		+				+		+		+	+	+			+			
11		+		+				+			+		+			+	+			+
12		+						+	+			+	+	+	+		+			
13		+		+				+					+	+	+	+	+	+		+
14		+		+				+					+	+		+	+			+
15		+		+				+					+	+	+	+	+	+		+
16		+		+				+					+			+				+
17		+		+								+	+			+	+			+
18		+		+				+						+	+	+		+		+
19	+						+							+			+		+	
20		+		+				+			+		+			+	+			+
21				+				+					+			+				+
22																	+			
23																	+			
24		+		+									+	+		+	+			
25		+		+				+		+			+	+			+			
26		+		+				+					+	+		+	+			+
27		+		+				+					+	+		+				+
28	+						+													
29								+												

+ *Indicates lysis.*

Lysis by a Composite Virus-filtrate

Because of the failure of any single-culture virus-filtrate to lyse all cultures of *X. trifolii*, a composite was prepared by mixing equal portions of virus-filtrates IV, VIII, XVII, and XIX. These were selected because collectively they lysed all the cultures with which virus-filtrates were obtained. When tested by the method used with single-culture virus-filtrates the composite lysed all 29 cultures shown in Table I, even culture 28, which was not lysed by any of the four single-culture virus-filtrates used in the composite. The composite virus-filtrate lysed 20 of 20 additional isolates from wheat: and 16 of 20 from barley, 15 of 20 from oats, 15 of 20 from flax, 20 of 20 from trefoil, 12 of 20 from brome grass, 19 of 20 from sweet clover, 14 of 20 from *Poa*, and 18 of 20 from wild oats. Likewise, the composite lysed 10 of 11 cultures selected on the basis of colony characteristics from a tryptone-glucose-extract agar plate prepared from raw milk. The milk had been plated in a routine laboratory exercise and the finding of these colonies was a chance one. The milk could have been contaminated from plant materials, which normally harbor these bacteria. It should be noted that only the original 29 cultures had been studied critically. The other 191 cultures tested were selected carefully on the basis of colony characteristics only. Unfortunately, the 32 cultures that were not lysed by the composite virus-filtrate were not checked to determine whether they differed from the original 29 in other characters also.

The composite virus-filtrate failed to lyse any of the 18 cultures of *X. translucens*. In addition, it failed to lyse *X. carotae*, *X. campestris*, *Sarcina lutea*, *Micrococcus citreus*, *M. pyogenes* var. *aureus*, *Agrobacterium radiobacter*, *Alcaligenes viscosus*, *Aerobacter aerogenes*, *Escherichia coli*, *M. pyogenes* var. *albus*, *Bacillus cereus*, *B. cereus* var. *mycoides*, *B. megatherium*, *B. subtilis*, *B. subtilis* var. *niger*, a mucoid variant of *B. subtilis*, and *B. sphaericus* var. *fusiformis*.

Titer of Virus-filtrates

Based on plaque counts on heavily inoculated nutrient agar plates the virus-filtrates ranged in titer from two hundred to four billion particles per milliliter. There was no apparent correlation between titer and number of cultures lysed by a virus-filtrate. As may be seen in Table I, virus-filtrate XII which contained four billion particles per milliliter lysed only four cultures, whereas virus-filtrate XVI with 200 particles lysed 13 cultures. Likewise there was no apparent correlation between titer and the number of transfers required to produce acceptable evidence of lysis. Each of virus-filtrates XII and XVI, referred to above, showed complete lysis at the second serial transfer from a single plaque. The effect of various storage conditions on titer is shown in Table II.

Discussion

The bacteria–virus relationship appears to be complex. On the accepted basis for grouping, the 20 cultures with which virus-filtrates were obtained

TABLE II

EFFECT OF STORAGE ON TITER OF VIRUS-FILTRATE

Virus	Weeks	Temperature, ° C.	Lysis
I	27	4	3+
II	28	4	3+
III	27	4	3+
IV	27	4	3+
V	31	4	2+
VI	35	4	1+
VII	22	4	2+
VIII	31	4	3+
IX	28	4	3+
X	28	4	1+
XI	35	4	2+
XII	22	4	2+
XIII	27	4	3+
XIV	32	4	3+
XV	32	4	2+
XVI	32	4	2+
XVII	34	4	3+
XVIII	32	4	2+
XIX	35	4	2+
XX	35	4	1+
Composite*	20	4	3+
Composite**	20	4	3+
Composite*	20	20	1+
Composite**	20	20	1+
III	27	−15	3+
VI	35	−15	1+
XII	22	−15	2+

* *Tested against culture 5.*
** *Tested against culture 13.*

represented one species: but on the basis of lysis by single-culture virus-filtrates no two cultures were identical. However, the 20 cultures had one common characteristic in that not one of the virus-filtrates from them lysed any of 18 cultures of *X. translucens*. Neither did the composite virus-filtrate lyse any of the 18, nor any of 17 other recognized species or subspecies.

This multiplicity of strains of virus probably was responsible for the failure of the composite virus-filtrate to lyse all isolates of what, without detailed study, were accepted to be representatives of *X. trifolii*. It is axiomatic that a composite virus-filtrate would lyse a greater number of cultures — be more inclusive — if it contained a greater number of single-culture virus-filtrates. For use in species identification, however, the inclusion of single-culture virus-filtrates must be limited to cultures meeting accepted requirements for species definition. The merit of the composite virus-filtrate used in this study relates to exclusiveness, rather than to inclusiveness.

The association of bacteria and virus on wheat probably represents a balance that is natural for a given sample, and although in this study the experimental evidence is not adequate to substantiate the theory, the low

numbers of these bacteria on certain samples of wheat probably are associated with relatively virulent viruses and the high numbers on other samples with less virulent viruses. Furthermore, the various strains of virus live in association without apparent antagonism. The 20 single-culture virus-filtrates were obtained from different portions of one sample of wheat merely by using different cultures, and the four strains used in the composite virus-filtrate retained their lytic property when mixed.

Acknowledgment

The authors are pleased to acknowledge the co-operation of Dr. W. A. F. Hagborg, Laboratory of Plant Pathology, Canada Department of Agriculture, Winnipeg, who provided the 18 cultures of *X. translucens* and carried out tests for pathogenicity on the 20 isolates of *X. trifolii*.

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THE SELECTIVE ENUMERATION OF ACTINOMYCETES IN THE PRESENCE OF LARGE NUMBERS OF FUNGI[1]

By C. T. Corke[2] and F. E. Chase[2]

Abstract

In response to a need for an actinomycete plating medium that would effectively inhibit fungi, two substances were tested — sodium propionate and the antibiotic cycloheximide (Acti-dione). Cycloheximide proved superior to the propionate. Five micrograms of cycloheximide per ml. of agar medium greatly reduced radial growth of fungal colonies, 40 μgm. almost completely prevented fungal growth, yet even 100 μgm. showed no inhibition of any of 85 actinomycete cultures isolated from agricultural and forest soil on media free of inhibitor. The concentration selected as most suitable for routine use was 40 μgm. of cycloheximide per ml. of agar medium, added just prior to the pouring of the plates.

Introduction

During current studies of the microbiology of acid forest-soils, it was found that enumeration and subsequent isolation of actinomycetes was often hampered by the extensive development of filamentous fungi on the agar medium. This is not surprising since fungi have frequently been observed to make up the dominant population in acid soils (5, 6), at times exceeding the combined numbers of bacteria and actinomycetes (1). Such interference during plating of bacteria and actinomycetes in agricultural soils seldom occurs, since fungi constitute a small part of the population and consequently are effectively removed by the preparation of serial dilutions. With acid forest-soil, on the other hand, the actinomycetes and bacteria will be diluted out first, so that some other means must be utilized to prevent the fungi from overgrowing dilution plates.

The use of fungal inhibitors was considered, and upon investigation two seemed promising. Crook *et al.* (2) reported that sodium propionate incorporated in glucose asparagine agar at a concentration of 0.4% inhibited fungi but allowed the growth of actinomycetes. Whiffen (8) stated that the antifungal antibiotic Acti-dione (referred to in this paper as cycloheximide) had no effect on the 11 bacterial species tested, even at a concentration of 1 mgm. per ml. of medium. Phillips and Hanel (4) found that this antibiotic showed no activity against any of the 42 bacterial strains examined (representing 27 species), and suggested that the addition of cycloheximide to media might be useful in the isolation of bacteria from materials contaminated with fungi. Since cycloheximide inhibits fungi without restricting bacteria, and since no reference to its effect on actinomycetes was found, an investigation of its possible value in preventing fungal interference when isolating actinomycetes was considered desirable.

[1]*Manuscript received September 27, 1955.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Canada. Presented in part at the Winnipeg meeting of the Canadian Society of Microbiologists in June, 1955.
[2]*Assistant and Associate Professors, respectively.*

Materials and Methods

Three media, widely used in the enumeration of actinomycetes, were selected as the basic media for this study, namely, egg albumen agar (7), dextrose asparagine agar (7), and glucose nitrate agar (3). Sodium propionate was added during preparation of the medium to give a final concentration of 0.4%. Cycloheximide* was prepared as a sterile stock solution (1 mgm. per ml. of distilled water filtered through a sterile ultrafine sintered glass filter), and portions were added aseptically to the melted agar media to produce the required concentration of antibiotic.

For plating studies, samples of the organic layer (A_o horizon) of acid forest-soils obtained from plots in the University of Toronto Forest near Algonquin Park, Ontario, were used, the pH of these samples varying from 3.5 to 5.0. Soil samples were diluted in sterile water blanks from an initial 1 : 10 dilution prepared aseptically with a Waring blendor by mixing for three minutes. In any one comparative trial five replicate plates were made of each treatment, all plates being inoculated from a single 1 : 10,000 dilution bottle. Plates were incubated at 27° C. for five days, when counts were made of the fungi, actinomycetes, and in some instances bacteria, developing on each plate. Microscopic examinations of colonies were made for group identification when necessary.

Results and Discussion

In a preliminary experiment, dextrose asparagine agar was prepared and divided into three portions. One portion was kept as control, the second was adjusted to contain 0.4% sodium propionate, and the third 10 μgm. of cycloheximide per ml. Examination after plating and incubation showed that the sodium propionate, while less effective in limiting fungal growth than cycloheximide, was more restrictive on the growth of actinomycetes. In fact, sodium propionate compared so poorly with cycloheximide that its use was discontinued. The results with cycloheximide, shown in Table I, indicate that at a concentration of 10 μgm. per ml. of medium, this compound, although not reducing the number of fungal colonies, did reduce their diameter to 8 mm. or less. This permitted a 10-fold increase in the number of actinomycete colonies, most of which could be easily isolated. The control plates for this experiment were completely overgrown with fungi and it was difficult to count the actinomycete colonies; furthermore, their isolation free of fungal contamination was impossible.

A comparative study was next made to determine the most suitable concentration of cycloheximide in each of the three media. Because the glucose nitrate medium of KenKnight and Muncie (3) consistently grew fewer actinomycetes than the other two, the results with egg albumen and dextrose asparagine agars only are presented in Figs. 1 and 2. As in the preliminary experiment, the fungi dominated the control media containing no inhibitor.

* *Cycloheximide for this study was kindly supplied by the Upjohn Company, Kalamazoo, Mich., U.S.A.*

TABLE I

EFFECT OF CYCLOHEXIMIDE ON NUMBERS OF ACTINOMYCETES AND FUNGI DEVELOPING ON DEXTROSE ASPARAGINE AGAR

(Average of five replicate plates, 10^{-4} dilution of forest soil)

Concentration of cycloheximide (μgm./ml. medium)	Actinomycetes	Fungi	Remarks
0	11	38	Fungi overgrew plates, making actinomycetes difficult to count
10	101	37	Fungal colonies were restricted to 8 mm. diam., most being less than 3 mm.; actinomycetes easily counted

The addition of 5 μgm. of cycloheximide per ml. of medium, although not appreciably reducing the number of fungal colonies, did restrict their radial growth so that the actinomycetes developing per plate increased from 2- to 10-fold, depending upon the medium. Additions of 10, 20, and 40 μgm. per ml. of agar medium resulted in a progressive reduction in the numbers of fungi that developed. Those fungal colonies which did grow in the maximum level of inhibitor were minute and showed aberrant hyphal development. The average actinomycete count increased with increasing concentration of cycloheximide, though in dextrose asparagine agar the difference between numbers of actinomycete colonies developing at 20 and 40 μgm. of inhibitor per ml. of medium was slight. Fig. 2 also indicates that the numbers of bacterial colonies developing on the plates of dextrose asparagine agar were doubled by the smallest addition of cycloheximide, with the higher concentrations giving small increases.

It was recognized that while the total numbers of actinomycetes developing on the plates were increased by increased concentrations of cycloheximide, yet the possibility existed that certain strains or species were being inhibited. To test this possibility, a total of 85 actinomycete cultures were isolated from agricultural and forest soil by random selection from agar plates containing no inhibitor. These cultures were then streaked onto agar plates containing cycloheximide at two levels, 50 and 100 μgm. per ml. of medium. Every one of the 85 cultures grew, with no evidence of inhibition. From this it was concluded that the danger of suppressing part of the actinomycete population by plating these forest or agricultural soils with media containing cycloheximide must be small, if it exists at all.

At present, in studies of both the bacterial and actinomycete populations of acid forest-soils, cycloheximide is incorporated in plating media at a level of 40 μgm. per ml. Our experience indicates that at this concentration cycloheximide effectively prevents fungal competition when we are studying microbial populations in acid forest-soils, where fungi often constitute a large

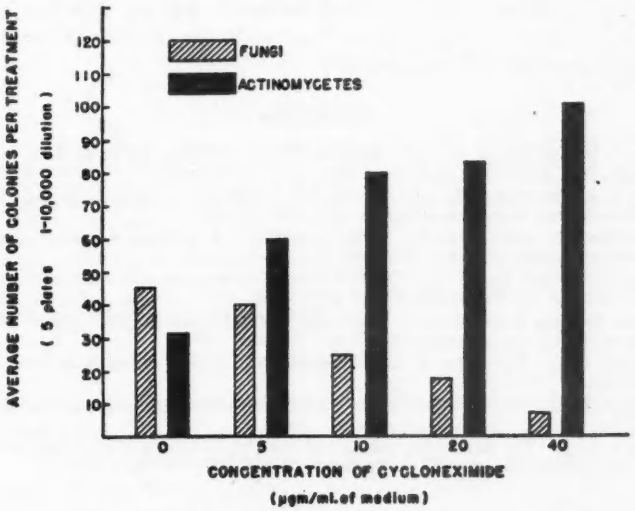

FIG. 1. Restriction by cycloheximide of fungal competition against actinomycetes in dilution plates of acid forest-soil on egg albumen agar.

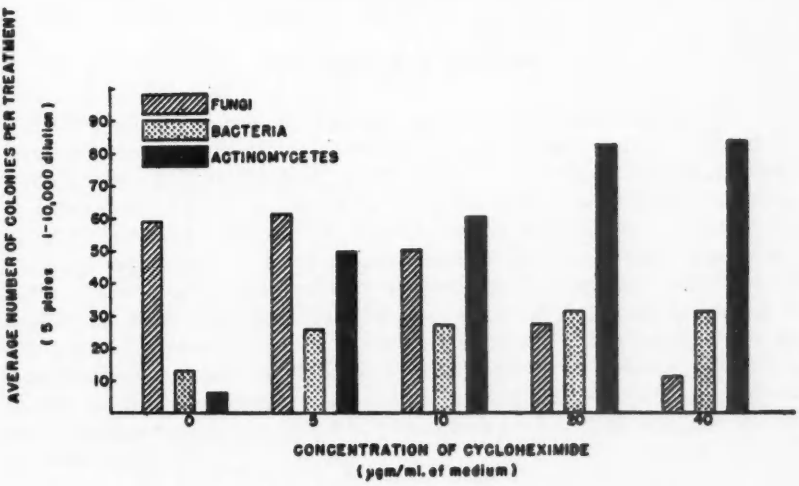

FIG. 2. Restriction by cycloheximide of fungal competition against actinomycetes and bacteria in dilution plates of acid forest-soil on dextrose asparagine agar.

part of the population. However, since fungi are seldom encountered in numbers comparable to bacteria and actinomycetes in soils having a pH above 6.0, which includes most agricultural soils, the addition of cycloheximide in these circumstances is not necessary.

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THE UTILIZATION OF DESOXYRIBONUCLEOTIDE PHOSPHORUS BY ESCHERICHIA COLI[1]

By S. M. Lesley and A. F. Graham

Abstract

The utilization of desoxyadenylic, desoxyguanylic, and thymidylic acid phosphorus by *E. coli* has been studied. Under comparable conditions phosphate is utilized from the three nucleotides at about the same rate. The rate of uptake is independent of nucleotide concentration, up to about 15 μgm. of nucleotide per ml., but markedly dependent on the inorganic phosphate concentration of the medium. *E. coli* can split off thymidylic acid phosphorus and release it into the medium as inorganic phosphate. Phosphate taken up from thymidylic and desoxyadenylic acids is distributed uniformly over the nucleotides of bacterial RNA and of DNA. It is concluded that nucleotide phosphate is assimilated exclusively as inorganic phosphorus.

Introduction

Exogenous purine and pyrimidine nucleotides have been found by several investigators (1, 2, 3, 8, 18) to be effective precursors of nucleic acid bases in *E. coli*. During these studies in our laboratory it was observed (8), in accordance with the results of Bolton (2), that phosphorus is split from the nucleotide during assimilation by the bacteria. Further study of the mechanism of nucleotide phosphorus utilization, the results of which are presented here, has shown that it is released into the medium as inorganic phosphorus by *E. coli*. Extracellular inorganic phosphorus concentration is the main factor determining the rate at which these bacteria assimilate phosphorus from the nucleotide.

Materials and Methods

Bacteria and Medium

Escherichia coli, strain B, was the test organism used throughout. The medium contained in grams per liter: $MgSO_4 \cdot 7H_2O$, 0.12; $CaCl_2$, 0.01; NH_4Cl, 0.44; KCl, 0.15; NaCl, 0.59; $FeCl_3$, 0.0003; Tris (hydroxymethyl)-aminomethane, 12.1; glucose, 4.0. Tris was kept as a 1 *M* stock solution, pH 7.7, and added to the salt solution before autoclaving. Glucose was sterilized separately as a 4% solution and added before each experiment. Since it was often desired to vary the inorganic phosphate content of the medium, a stock solution of Na_2HPO_4 was sterilized separately and added in the required amount just prior to each experiment. Each bacterium in the logarithmic growth phase contains 8.3×10^{-9} μgm. of phosphorus. Therefore, 15 μgm. of inorganic phosphorus per ml. of medium should support logarithmic multiplication to a density of 1.8×10^9 bacteria per ml. This has been verified by experiment.

[1]*Manuscript received October 21, 1955.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. Supported by grants from the National Cancer Institute of Canada; The National Institutes of Health of the United States, Public Health Service; and the W. B. Boyd Memorial Fund.

Enzymes

Pancreatic desoxyribonuclease (DNAse) was obtained commercially as the twice crystallized enzyme. Phosphodiesterase was prepared from dried snake venom (*Agkistrodon piscivorus*) by the chromatographic technique described by Hurst and Butler (9). Sodium chloride, 0.03%, was used to elute the enzyme from the filter paper column. Phosphodiesterase thus prepared contained a negligible amount of monoesterase.

Propagation of Bacteria

Bacteria were maintained by daily subculture in the defined medium. These cultures were agitated continuously at 37° C. For all experiments cultures were shaken vigorously at 37° C. in vessels of the type described by Siminovitch (manuscript in preparation). The generation time in all experiments was determined by frequent turbidity measurements at 425 mμ in a Fisher Electrophotometer. Optical densities on the spectrophotometer were translated into cell concentrations with a curve standardized by plate count. Under the conditions used here the bacterial generation time was 54 to 56 min.

Preparation of Desoxyribonucleotides

About 10 gm. of DNA (11) were dissolved in water, heated to 100° C. for 10 min., and the solution was treated successively with DNAase and phosphodiesterase as described by Hurst, Marko, and Butler (10). The resulting mononucleotides were separated by placing the solution, at pH 9, on a column (170 cm. × 20 cm².) of Dowex-1 ion exchange resin in the acetate form and eluting it with acetate buffers (10). Each nucleotide was precipitated as the barium salt and kept *in vacuo* over calcium chloride.

When free nucleotide was required, a concentrated solution of the barium salt in water, at pH 9, was run through a small column of Dowex-50 cation exchange resin in the sodium form. The column was washed with 5 ml. of distilled water and the two effluents combined. Recovery of the mononucleotide was almost quantitative and the solution was free of barium. Purity of the nucleotide was determined by subjecting an aliquot of the solution to electrophoresis on filter paper, saturated with 0.05 *M* ammonium formate buffer, pH 3.5, for 90 min. at 20 volts per cm. (4, 15). No nucleoside or base impurities were observed. This test is a sensitive one since the nucleotide migrates to the anode while nucleosides and bases move toward the cathode or remain at the starting line.

Molecular extinction coefficients were determined for the desoxyribonucleotides prepared in the above manner. The optical density of a nucleotide, in 0.1 *N* hydrochloric acid, was determined at the wavelength of maximum absorption on a Beckman DU Spectrophotometer. Phosphorus estimations were carried out on the nucleotide solution by the method described elsewhere (7), and molecular extinction coefficients were calculated on the assumption that all the phosphorus was nucleotide phosphate. These results are given in Table I. The ribonucleotide coefficients used were those given by Deluca, Rossiter, and Strickland (5).

TABLE I

MOLECULAR EXTINCTION COEFFICIENTS OF DESOXYRIBONUCLEOTIDES IN 0.1 *N* HYDROCHLORIC ACID

Nucleotide	Molecular extinction coefficient
Desoxyadenylic acid	13900
Desoxyguanylic acid	12300
Desoxycytidylic acid*	12100
Thymidylic acid	9300

** Probably contains a small amount of 5-methyldesoxycytidylic acid since this was not separated by the ion exchange chromatography.*

Preparation of P^{32} Labelled Desoxyribonucleotides

Desoxyribonucleotides labelled with P^{32} were prepared from T2 phage labelled and purified in the manner described previously (12). A suspension of labelled phage, containing about 20 mgm. of DNA, in 5 ml. of 0.8% sodium chloride, was heated to 100° C. for 10 min. DNAase and phosphodiesterase were added and the resulting mononucleotides separated on a column of Dowex-1 acetate as described in the preceding section. To concentrate the nucleotides after elution from the ion exchange column their solutions were passed through short columns of activated charcoal. These columns were washed with water and the nucleotides eluted in small volumes of ammoniacal ethanol.* The solutions thus obtained were evaporated to dryness at 50° C. in a current of nitrogen and the nucleotides dissolved in small volumes of distilled water. To estimate the nucleotide content of a solution an aliquot was diluted in 0.1 *N* hydrochloric acid, its optical density determined at the wavelength of maximum absorption, and the amount calculated from the extinction coefficient. Radioactivity assays were carried out in a 10 ml. liquid counter of the type described by Veall (19).

Separation of the Nucleotides from Bacterial RNA and DNA

The following method was used to separate the nucleotides of RNA and DNA from bacteria labelled with P^{32} and determine their specific activities. Cold 50% TCA (trichloracetic acid) was added to the chilled culture to give a final concentration of 5%. After 15 min. at 5° C. the precipitated cells were centrifuged, washed once with cold 5% TCA, twice with cold absolute ethanol, and boiled twice for 15 min. periods with absolute ethanol. The residue was evaporated just to dryness at 50° C. under nitrogen and 6.0 ml. of 0.3 *N* sodium hydroxide added (17). After incubation for 18 hr. at 37° C. the solution was chilled, the pH adjusted to about 7 with dilute HCl and DNA precipitated by addition of cold TCA to 5% concentration.

After it was centrifuged, the supernatant containing RNA was adjusted to pH 4.5 with acetate buffer and the mononucleotides adsorbed to and eluted

** We are indebted to Dr. C. W. Helleiner, Biochemistry Department, University of Toronto, for describing the details of this technique to us before publication.*

from a small column of activated charcoal as described in the previous section. The eluate was evaporated to a small volume under nitrogen at 50° C., and the mononucleotides were separated by ion-exchange chromatography on Dowex-1 acetate resin. Each nucleotide was concentrated and freed from salt by adsorption to and elution from activated charcoal and then subjected to electrophoresis on paper at pH 3.5. After elution from the paper optical densities (in 0.1 *N* hydrochloric acid) and P^{32} estimations were carried out and the specific activities computed.

The sediment containing DNA was washed once with cold 5% TCA, twice with cold ethanol saturated with sodium acetate, and dissolved in a small volume of Tris buffer, 0.01 *M*, pH 7.5. Mononucleotides were obtained by successive digestion with DNAase and phosphodiesterase. Acetic acid was added to bring the pH to 4.5 and the mononucleotides were adsorbed to and eluted from activated charcoal. The volume of eluate was reduced at 50° C. in nitrogen and the mononucleotides separated by electrophoresis on paper at pH 3.5. Each nucleotide was eluted from the paper with water and subjected to two dimensional paper chromatography. Isobutyric acid (163 ml.), water (96.7 ml.), ammonium hydroxide (S.G. 0.880, 3.3 ml.) were used in the first dimension (14), and ethanol (95%, 75 ml.) and *M* ammonium acetate (pH 7.5, 30 ml.) in the second (16). Areas of paper containing the nucleotides, and suitable blank areas, were eluted with water. Specific activities were determined in the same manner as for the RNA nucleotides.

Results

Uptake of Nucleotide Phosphate by E. coli

The following experiment shows that desoxyadenylic acid phosphorus is assimilated by *E. coli* in the logarithmic growth phase. A culture of bacteria was grown to a concentration of 1.5×10^9 cells per ml.; the number of cells increased at a logarithmic rate throughout with a generation time of 52 min. An aliquot of these bacteria was diluted in fresh medium to give 5×10^8 cells per ml. and P^{32} labelled desoxyadenylic acid was added to a final concentration of 2 μgm. per ml. This culture was then vigorously shaken at 37° C., cell multiplication being determined turbidimetrically. At intervals, duplicate 1.0 ml. aliquots were removed and treated in the following manner. One sample was added to 8.0 ml. of cold phosphate buffer, pH 7, containing a small amount of crystalline egg albumin as protein carrier. One milliliter of cold 50% TCA was then added; after it had been allowed to stand 20 min. at 5° C., the mixture was centrifuged and the supernatant assayed for P^{32}. The duplicate sample was added to 9.0 ml. of a chilled suspension of carrier cells (about 2×10^8 per ml.) containing formaldehyde at a concentration of 1.8%. After 15 min. in the cold the cells were removed by centrifugation and P^{32} determined in the supernatant. From the growth curve of the culture the number of bacteria in each sample was determined and the amount of P^{32} per cell calculated. This value was plotted against time as shown in Fig. 1.

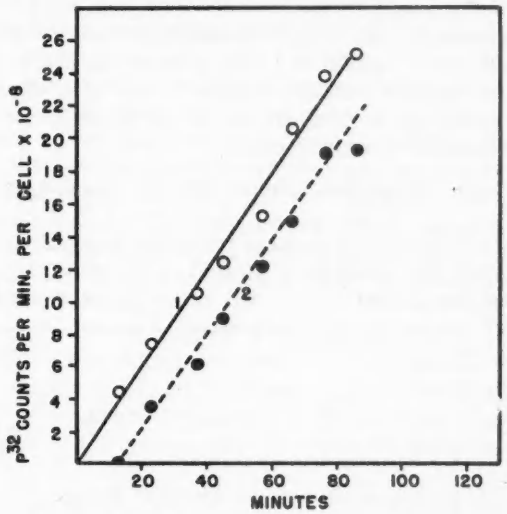

FIG. 1. Uptake of P^{32} from labelled desoxyadenylic acid during logarithmic growth of *E. coli*, in counts per min. per bacterium. Curve 1, determined after formaldehyde treatment of cells. Curve 2, determined after TCA treatment of cells. Utilization of nucleotide phosphate in 86 min. was 78%, Curve 1; and 59%, Curve 2. At the beginning of the experiment the medium contained 10 μgm. of inorganic phosphate per ml.

The technique following formaldehyde treatment permits an estimate of the total P^{32} taken up by the cell, while TCA precipitation allows an estimate of P^{32} incorporated into acid insoluble constituents. Both techniques yield the same rate for assimilation of nucleotide phosphate by the cell, although one curve lags behind the other by about 15 min.

When similar experiments were carried out with labelled desoxyguanylic or thymidylic acids in the medium, P^{32} was taken up by the bacteria at about the same rate as from desoxyadenylic acid.

Influence of Nucleotide Concentration on Assimilation of Nucelotide Phosphorus

It was next of interest to determine what influence the concentration of exogenous nucleotide had on the rate of uptake of its phosphorus by the cell. Four cultures of *E. coli* were prepared each containing 5×10^8 bacteria, 0.6 μgm. of P^{32} labelled desoxyadenylic acid, and 10 μgm. inorganic phosphorus per ml. Each culture also contained unlabelled desoxyadenylic acid so that the final concentrations of nucleotide were 5, 10, 15, and 20 μgm. per ml. respectively. The cultures were agitated at 37° C., samples being removed at intervals and precipitated with TCA as before. After centrifuging, the sediments were washed twice with cold 5% TCA, dissolved in 0.1 *N* sodium hydroxide, and assayed for P^{32} content. The results obtained with each culture were calculated as c.p.m. of P^{32} per cell and plotted against time as shown in Fig. 2.

Up to a concentration of 15 μgm. of nucleotide per ml. the difference in rates is probably not significant. The amount of nucleotide phosphate incorporated into the acid insoluble fraction of the cell is, therefore, approximately proportional to the concentration of nucleotide in the medium when the amount is less than 15 μgm. per ml.

Effect of Inorganic Phosphorus Concentration on Assimilation of Nucleotide Phosphate

One striking feature of the kinetics of nucleotide phosphorus uptake, shown both in Fig. 1 and Fig. 2, was their linearity. It can be shown (13) that, if nucleotide were assimilated by the cell as an intact molecule the rate of

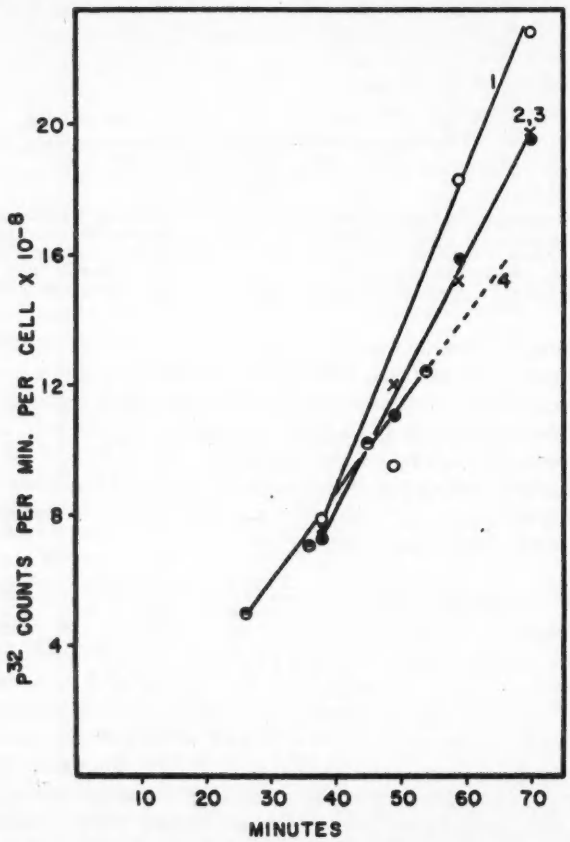

FIG. 2. Effect of nucleotide concentration on the uptake of P^{32} from labelled desoxyadenylic acid during logarithmic growth of *E. coli*. Total nucleotide concentrations were 5, 10, 15, and 20 μgm. per ml. in experiments corresponding to Curves 1, 2, 3, and 4 respectively. Each culture contained 0.6 μgm. of labelled nucleotide. Utilization of nucleotide phosphate in 70 min. was 57%, Curve 1; 49%, Curve 2; 48%, Curve 3 and 25%, Curve 4.

nucleotide phosphorus uptake should equal $K(1\text{-}e^{\alpha t})$ c.p.m. per cell, where K is a constant, α the generation time, and t is time. Under the conditions prevailing in our experiments the kinetic curves should then have been significantly concave to the time axis rather than linear. It was possible, however, to account for the linear uptake kinetics by assuming that, prior to its assimilation by the cell, nucleotide phosphorus was converted to inorganic phosphate and the latter was in equilibrium with inorganic phosphate in the medium. This hypothesis will be discussed later but it suggested that the rate of utilization of nucleotide phosphate should depend on the concentration of inorganic phosphate in the medium. That such is the case is shown by the following experiment.

Two bacterial cultures were prepared, each containing 5×10^8 cells and 0.6 μgm. of P^{32} labelled thymidylic acid per ml. In one culture the inorganic phosphorus concentration was 15 μgm. per ml., in the other 26 μgm. per ml. Assimilation of P^{32} was followed by measuring the increase in acid insoluble isotope during growth of the culture, and the results are shown in Fig. 3.

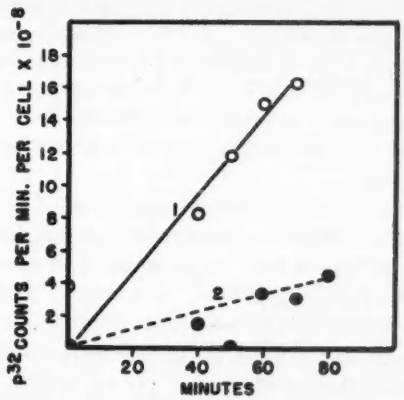

FIG. 3. Effect of inorganic phosphate concentration on the uptake of P^{32} labelled thymidylic acid phosphorus during logarithmic growth of *E. coli*. Each culture contained 0.6 μgm. of labelled thymidylic acid per ml. In cultures corresponding to Curves 1 and 2 inorganic phosphorus concentrations were respectively 15 and 26 μgm. per ml. Utilization of nucleotide phosphate in 70 min. was 53%, Curve 1; and 13%, Curve 2.

It is apparent that the rate of assimilation of nucelotide phosphate is reduced by increasing the amount of inorganic phosphate. The magnitude of the effect is sufficiently large to suggest that most of the nucleotide phosphorus was utilized as inorganic phosphate.

Site of Hydrolysis of Nucleotide Phosphate

Removal of nucleotide phosphorus could be effected by the organism itself as a metabolic step in its assimilation of nucleotide, or could occur extracellularly as the result of a phosphomonoesterase elaborated by the bacteria during multiplication. The following experiment demonstrates that a cell

free extract contains an enzyme able to hydrolyze thymidylic acid. Seven milliliters of a bacterial culture containing 1.5×10^8 cells per ml. were "snap lysed" by the addition of potassium cyanide to 0.01 *M* concentration and about 100 T2 phages per bacterium (6). After centrifuging, 5.0 ml. of the extract was incubated at 37° C. with 1.3 μgm. of P^{32} labelled thymidylic acid per ml. Aliquots were removed at intervals and the extent of nucleotide hydrolysis determined in each. To do this a relatively large and constant amount of unlabelled thymidylic acid was added to each sample. Nucleotide concentrations were then estimated spectrophotometrically and from these, and P^{32} determinations, the specific activities were calculated. It should be noted that the final concentration of *labelled* nucleotide was too small to be estimated spectrophotometrically but the P^{32} concentration could be readily measured. Part of each solution was then subjected to electrophoresis on paper saturated with 0.05 *M* ammonium formate buffer, pH 3.5, for 60 min. at 20 volts per cm. This was sufficient time to separate any inorganic phosphate or desoxyribose phosphate from unhydrolyzed nucleotide. The area of paper containing nucleotide and a neighboring blank area of equal size were shaken with water for 60 min. at 37° C. Determinations of nucleotide and P^{32} concentrations were done and the specific activity of the nucleotide calculated. Comparison of this figure with the apparent specific activity of the nucleotide before paper electrophoresis indicated that 7.5% of the labelled thymidylic acid had been hydrolyzed by the bacterial extract after 30 min. and 48% after 90 min.

As a control to the above experiment a preparation of labelled thymidylic acid of known specific activity was subjected to paper electrophoresis. After elution of the thymidylic acid from the paper estimation of its specific activity showed that 6% had been hydrolyzed. This may be the error of the method or the amount of nonnucleotide phosphorus in the thymidylic acid preparation.

In a further experiment a bacterial culture containing 5×10^8 cells and 1.6 μgm. of P^{32} labelled thymidylic acid per ml. was incubated for 20 min. at 37° C. and centrifuged. Using the electrophoresis technique described above, 56% of the P^{32} remaining in the supernatant was found to be no longer associated with thymidylic acid. Thus the nucleotide is hydrolyzed by a bacterial culture and its phosphorus appears in the medium.

Hydrolysis of nucleotide did not occur in the medium as a result of an enzyme released during multiplication of *E. coli* as shown in the following experiment. A culture was grown to 1.6×10^8 cells per ml. and centrifuged. The supernatant was incubated for 120 min. with a small concentration of labelled thymidylic acid and the amount of hydrolysis determined. There was no breakdown of the thymidylic acid.

It was concluded from these experiments that the phosphorus of exogenous thymidylic acid is removed by the bacteria during logarithmic growth of *E. coli* and released into the medium as inorganic phosphate. The cells contain an enzyme able to effect this dephosphorylation.

Incorporation of Nucleotide Phosphorus into Bacterial Nucleic Acids

While the foregoing results indicated that nucleotide phosphorus was largely assimilated as inorganic phosphate it was possible that some nucleotide was also used as the intact molecule. Information on this point was obtained by propagating *E. coli* in the presence of P^{32} labelled nucleotides and then determining the specific activities of the nucleotides in bacterial RNA and DNA. If all nucleotide phosphorus were assimilated as inorganic phosphate, the cellular nucleic acids should be uniformly labelled. But if, for example, 10% of exogenous thymidylic acid were incorporated unchanged into bacterial nucleic acid, the specific activity of cellular thymidylic acid should be about twice that of any other constituent nucleotide. Table II shows the results of such experiments when bacteria were grown in the presence of P^{32} labelled thymidylic or desoxyadenylic acid.

The results demonstrate that both ribo- and desoxyribonucleotides of the bacterial nucleic acids are uniformly labelled. It is concluded that exogenous nucleotide phosphorus is assimilated almost exclusively as inorganic phosphate. It is also evident that ribonucleotides attain a significantly higher specific activity than the desoxyribonucleotides. This observation is in accord with other results in this laboratory (L. Siminovitch, manuscript in preparation) that inorganic phosphate is incorporated into bacterial RNA at a faster rate than into DNA.

TABLE II

DISTRIBUTION OF ASSIMILATED NUCLEOTIDE PHOSPHORUS IN THE NUCLEIC ACIDS OF *E. coli*

	Specific activity, c.p.m. per μgm. P	
	Experiment 1	Experiment 2
Bacterial RNA		
Adenylic acid	25	17
Guanylic acid	25	15
Cytidylic acid	29	20
Uridylic acid	30	16
Bacterial DNA		
Desoxyadenylic acid	21	8
Desoxyguanylic acid	24	7
Desoxycytidylic acid	22	8
Thymidylic acid	24	8

Experiment 1. Bacteria were grown in 100 ml. of medium containing 7 μgm. of inorganic phosphorus per ml. When the concentration reached 5×10^8 cells per ml. a tracer amount of P^{32} labelled thymidylic acid was added. When the bacterial concentration reached 7.5×10^8 per ml. 40% of the P^{32} had been assimilated by the cells. A further 4 μgm. of inorganic phosphorus per ml. was then added and growth of bacteria continued exponentially to 1.1×10^9 cells per ml. At this point 250 ml. of unlabelled carrier bacteria, containing 1.5×10^9 cells per ml. in the exponential phase was added, and the resulting culture was immediately used for nucleotide analysis.

Experiment 2. Bacteria were grown in 30 ml. of medium containing 15 μgm. of inorganic phosphorus per ml. and a tracer amount of P^{32} labelled desoxyadenylic acid. Growth continued in the exponential phase to 1.5×10^9 cells per ml. when 40% of the P^{32} had been taken up by the cells. At this point 300 ml. of unlabelled carrier bacteria, at a concentration of 1.5×10^9 cells per ml., was added and the resulting culture used immediately for nucleotide analysis.

Discussion

The present results with those of Bolton (2) and Balis *et al.* (1) demonstrate that nucleotides are not assimilated as intact molecules by *E. coli*. Nucleotide phosphate is split off, released into the medium as inorganic phosphate, and the rate of its subsequent utilization is dependent on the concentration of extracellular inorganic phosphate. This explains the lack of nucleotide phosphorus utilization in Bolton's experiments (2) since the inorganic phosphorus content of his medium was about 2 mgm. per ml. There was some uptake in the experiments of Balis *et al.* (1) in spite of a high concentration of medium inorganic phosphate, but their cultures were propagated for prolonged periods in the presence of nucleotide.

It is evident that in our experiments two concurrent processes contribute to the rate of nucleotide phosphate uptake. Firstly, the medium is being depleted of inorganic phosphate at a rate consistent with logarithmic growth of the bacteria. Secondly, nucleotide P^{32} is continually being released into the medium. Consequently, the specific activity of medium inorganic phosphorus increases in a complex manner and results in a linear rate of P^{32} uptake, during one to two bacterial generations at least.

Since the intracellular fate of the nucleotide carbon skeleton is probably different from that of assimilated nucleoside (8, 18), removal of the phosphate must be a necessary metabolic step in utilization of nucleotides by *E. coli*. On the other hand dephosphorylation of an exogenous nucleotide is not necessarily followed by assimilation of the nucleotide carbon; thymidylic acid is dephosphorylated by *E. coli* as rapidly as other desoxyribonucleotides but its carbon moiety is little, or not at all, utilized for bacterial nucleic acid synthesis (18).

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MICROBIAL PENTOSANASES

II. SOME FACTORS AFFECTING THE PRODUCTION OF PENTOSANASES BY BACILLUS PUMILUS AND BACILLUS SUBTILIS[1]

By F. J. Simpson

Abstract

A number of carbohydrates and nitrogenous adjuncts were tested for their effect on the constitutive and adaptive pentosanases produced by *Bacillus subtilis* and *B. pumilus* respectively in a medium containing biotin, ammonium phosphate, and other mineral salts. *B. subtilis* produced more enzyme with sulphite liquor than with any of the other carbohydrate sources tested. Next, in decreasing order of merit, were wheat bran, maltose, ribose, beet molasses, oat hulls, and pectin. Of the nitrogenous adjuncts tested, corn steep liquor, soybean meal, gelatin, gelysate, and ammonium lactate doubled the yield of enzyme whereas yeast extract, peptone, urea, and others were less effective. For *B. pumilus* the better carbohydrate sources, in decreasing order of merit, were wheat bran, water soluble pentosan of wheat flour, xylan, straw holocellulose, wheat straw, and sulphite liquor. Of the nitrogen sources, corn steep liquor was outstanding while casein, casitone, phytone, yeast extract, distillers' dried solubles, and soybean meal followed in decreasing order. A medium containing 6% wheat bran (20 mesh), 1% corn steep liquor neutralized with ammonia, 0.05% sodium chloride, and 0.05% calcium carbonate was devised for the production of pentosanase by *B. pumilus*. With this medium in shaken Erlenmeyer flasks, the enzyme was produced at a high rate between 12 and 40 hr.; thereafter the rate of production decreased. Maximum yields were obtained in 96 hr. A temperature of 26° C. was more favorable for pentosanase production than higher temperatures.

Introduction

Pentosanases produced by *Bacillus pumilus* and *Bacillus subtilis* have been used successfully to hydrolyze the pentosans of "squeegee starch" with resultant increases in the amount of starch recovered in the process of separating starch and gluten from wheat flour (8). These enzymes may prove helpful in other processes where pentosans cause difficulties such as in reducing the viscosity of malt worts. They may also assist the carbohydrate chemist in his studies on the structure of pentosans. In the present investigation, some factors affecting the production of pentosanases by the two bacilli were studied. Since *B. pumilus* produces pentosanase but not amylase, this organism was studied in more detail and a medium suitable for large scale production of the enzyme was devised.

Materials and Methods

The strain of *B. pumilus*, PRL B12, and the strain of *B. subtilis*, PRL B92, used in this study had previously been found to produce extracellular pentosanases in good yield (2, 7). These strains were stored in sterilized soil. Inoculum was obtained by transferring a loopful of soil to 50 ml. of medium in a 250 ml. Erlenmeyer flask and incubating it for 16 to 24 hr. at 30° C. on

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a Gump rotary shaker (200 r.p.m., 1¼ in. eccentricity). The two ingredients of the medium, Difco nutrient broth (0.4%) and sugar (0.5%), were sterilized separately and aseptically combined. Xylose was used for *B. pumilus* and glucose for *B. subtilis*.

One-half a milliliter of inoculum was used to inoculate 50 ml. of test medium in Erlenmeyer flasks of 500 ml. capacity. The use of larger volumes of test media per flask resulted in lower yields. The basal test medium to which various carbohydrates and nitrogenous materials were added consisted of 0.4% $(NH_4)_2HPO_4$, 0.1% KCl, 0.2% $CaCO_3$, 2 μgm. biotin per ml., and 0.103% of a salt mixture consisting of 1 gm. $MgSO_4 \cdot 7H_2O$, 0.01 gm. $ZnSO_4 \cdot 7H_2O$, 0.005 gm. $CuSO_4 \cdot 5H_2O$, 0.0075 gm. $MnSO_4 \cdot H_2O$, and 0.01 gm. $FeSO_4 \cdot 7H_2O$. Usually the basal medium and the nitrogenous supplements were sterilized together and then an aliquot of a sterile solution of the carbohydrate aseptically added to give a final concentration of 2%. Those carbohydrates that did not lend themselves to this technique, such as the pectic substances, xylan, holocellulose, straw, bran, pentosan of wheat flour, and oat hulls, were sterilized with the basal medium.

The flasks containing test media were incubated at 30° C. on the rotary shaker unless otherwise indicated in the text. Samples of approximately 2 ml. were withdrawn aseptically at 40 hr. and a final sample at 64 hr. The pH was determined and after removal of bacterial cells and insoluble matter in the centrifuge the pentosanase activity was determined on suitable dilutions of the supernatant liquor by the procedure previously described (7).

Care was taken to see that the pH during the fermentations did not fall below the point (pH 5.5) where pentosanase is rapidly destroyed. This was not difficult in the presence of 0.2% $CaCO_3$ and an organic nitrogenous supplement such as yeast extract or ammonium lactate. In the absence of a nitrogenous supplement it was necessary to double the amount of $CaCO_3$ for *B. pumilus* as this organism produced more acid than *B. subtilis*. Each test was replicated four or five times and the least significant difference (L.S.D.) was calculated (3).

Sulphite waste liquor was treated by the method developed by Murphy and Stranks (4). The liquor was boiled and aerated for 15 min., concentrated under vacuum to half its original volume, adjusted to pH 8.5 with ammonia, and 25 ml. aliquots sterilized in Erlenmeyer flasks. To this was added 25 ml. of sterile double strength basal medium. The xylan was a commercial preparation purchased from Pfanstiehl. The pentosan of wheat flour was the water soluble preparation previously described (7). The oat hulls and wheat bran were purchased from a local milling firm. Although the wheat bran was contaminated with a small amount of buckwheat, no difference in pentosanase production was observed between this contaminated bran and that from which buckwheat was removed manually. The bran contained 2.6% nitrogen, 1.1% phosphorus, and 26.6% pentosan. The wheat bran, oat hulls, and wheat straw were ground in a Wiley mill to pass a 20 mesh sieve. The beet molasses which contained 52% sucrose as determined after acid hydrolysis

by the method of Somogyi (10) was diluted to give a final concentration of 2% sucrose. The corn steep liquor contained 53% solids and 4.07% nitrogen, and was adjusted to pH 6.5 with concentrated NH_4OH (*ca.* 8 ml. per 100 gm.). The casein hydrolyzate was an acid hydrolyzed vitamin free preparation (Nutritional Biochemical Corporation).

Nitrogen was determined by the semimicro Kjeldahl method with a copper–selenium catalyst, and pentosan by the method of Adams and Castagne (1).

Experimental

The Effect of Temperature on the Production of Pentosanases

Three Gump rotary shakers in separate rooms controlled at 26°, 30°, and 35° C. were used to agitate the Erlenmeyer flasks, while a New Brunswick shaker (1 in. eccentricity) was used for 40° C. Fifty milliliters of basal medium supplemented with 0.5% yeast extract was inoculated with 0.5 ml. of a 16-hr. inoculum and placed on the shakers. At intervals triplicate flasks were removed, water added to compensate for that lost by evaporation, and the pH and pentosanase activities determined. With *B. pumilus* the pH throughout these fermentations was fairly steady, dropping from an initial pH of 6.4 to 6.0 and then rising to a final pH of 7.0 at the lower temperatures and to 7.4 at 40° C. The pH values for the fermentations with *B. subtilis* rose rapidly from pH 6.4 to 7.6–7.8 and remained there. Under these conditions both organisms produced the maximum pentosanase at 26° C. although *B. subtilis* produced an equal amount at 30° C. (Figs. 1 and 2). The higher yield of enzyme at 26° C. may be related to greater stability at this temperature. Both organisms grew well at the higher temperatures even though pentosanase production was apparently reduced. Unfortunately, it was not possible to conduct tests at temperatures lower than 26° C. In addition, as the shakers were being employed for other investigations, subsequent tests were made at 30° C.

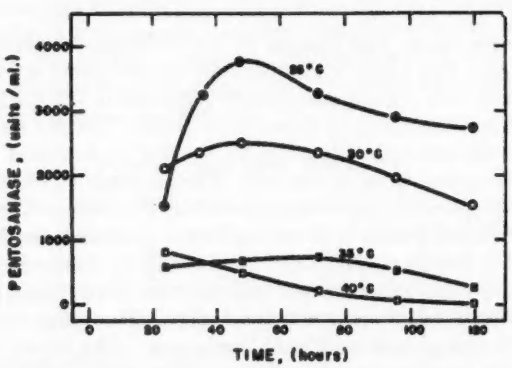

FIG. 1. The effect of temperature on production of pentosanase by *B. pumilus* in a medium containing 2% xylose, 0.5% yeast extract, biotin, and mineral salts.

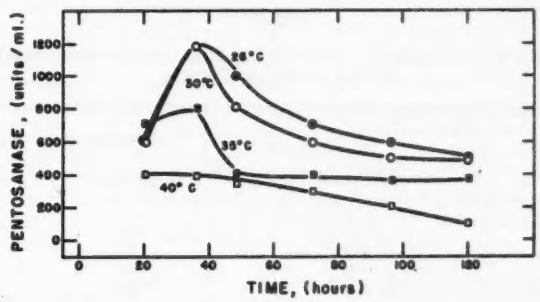

FIG. 2. The effect of temperature on production of pentosanase by *B. subtilis* in a medium containing 2% xylose, 0.5% yeast extract, biotin, and mineral salts.

Comparison of Nitrogenous Supplements

Compared with other media devised for the production of enzymes, the basal medium is low in available nitrogen. For example, Shu (5), in studying the production of amylase by *Aspergillus niger*, obtained optimum yields with a nitrogen to carbohydrate ratio of 1 : 13, and similarly good yields of amylase were obtained with *Streptomyces griseus* in a medium with a ratio of 1 : 17 (9). The nitrogen to carbohydrate ratio in the basal medium is 1 : 23. Accordingly, a number of inorganic and organic supplements high in nitrogen were added to the basal medium, not only to determine whether additional nitrogen would increase the yield of enzyme, but also to determine if certain forms of nitrogen might be more beneficial than others. The supplements were added in concentrations equal to the addition of 0.5 gm. of nitrogen per liter. Xylose (2%) was used as the substrate for *B. pumilus* and glucose for *B. subtilis*.

B. pumilus did not produce more enzyme in the presence of additional inorganic nitrogen (Table I). Although the addition of the ammonium salts of lactic, citric, or succinic acids did stimulate the production of pentosanase, the greatest stimulation was obtained with protein hydrolyzates, proteins, and fermentation waste products. Of these, corn steep liquor was outstanding. Since the pH of all the fermentations was well within the range 5.9 to 7.5, the supplements, in addition to their possible effect on maintaining a stable pH in the immediate vicinity of the bacterial cells (5), must be contributing substances other than biotin that stimulate enzyme production either directly or indirectly by way of improved growth.

The effect of additional nitrogen on the production of pentosanase by *B. subtilis* was not as distinctive as with *B. pumilus*. Of the supplements tested, ammonium lactate, gelysate, phytone, peptone, casein, gelatin, soybean meal, and corn steep liquor were most beneficial and about equal in effect. Both *B. pumilus* and *B. subtilis* evidently secrete potent proteolytic enzymes for the response to protein hydrolyzates is no greater than to the proteins themselves.

TABLE I

EFFECT OF NITROGENOUS ADJUNCTS ON THE YIELD OF PENTOSANASE AFTER 64 HR. OF GROWTH

Material	Concentration, %	Pentosanase, units/ml.	
		B. pumilus	*B. subtilis*
Control	—	100	556
$(NH_4)_2HPO_4$	0.236	100	542
NH_4Cl	0.196	90	421
KNO_3	0.362	54	508
Urea	0.106	60	513
(NH_4) acetate	0.275	100	654
(NH_4) lactate	0.472 (by vol.)	200	1010
NH_4 citrate	0.403	905	728
NH_4 succinate	0.272	420	650
Casein hydrolyzate	4.76 (by vol.)	1000	872
Casitone	0.458	2100	897
Gelysate	0.322	852	1100
Phytone	0.625	2110	968
Peptone	0.333	1450	970
Isoelectric casein	0.350	2000	1000
Gelatin	0.360	990	1220
Soybean meal	0.781	1680	1060
Yeast extract	0.532	1730	846
Corn steep liquor	1.23	3220	1080
Distillers' dried solubles	1.19	1690	912
Malt sprouts	1.04	1060	865
L.S.D. ($P = 0.05$)	—	237	205

Medium: 2% carbohydrate, 0.4% $(NH_4)_2HPO_4$, 0.1% KCl, 0.2% $CaCO_3$, biotin and nitrogenous supplement equivalent to 0.05 gm. N per liter.

Comparison of Carbon Sources

Bacillus pumilus possesses an adaptive pentosanase since glucose, galactose, and arabinose did not induce the production of pentosanase, but xylose and the water soluble pentosan isolated from wheat flour did (7). In addition to these substances, a number of other sugars and some complex carbohydrates known to contain pentosan were tested to determine their suitability as substrates for the production of pentosanases.

The inoculum for this survey consisted of 0.5 ml. of a 15-hr. culture of the organisms grown on 0.4% nutrient broth. The carbohydrates were added to the basal medium at a concentration of 2%, except beet molasses which was diluted to give a final concentration of 2% sucrose, and sulphite liquor which was used at normal strength.

B. pumilus again produced pentosanase only on those substrates which contained xylose or xylans (Table II). Wheat bran was the best of these. This may not only be a direct result of its high xylan content, but also because it contains other nutritive substances.

B. subtilis apparently possesses a constitutive pentosanase (7), however production of the enzyme is nevertheless stimulated by sugars such as arabinose, ribose, and maltose, and by a number of the complex carbohydrates

TABLE II

COMPARISON OF CARBON SOURCES FOR PRODUCTION OF PENTOSANASE

Carbohydrate	Pentosanase, units/ml.	
	B. pumilus	*B. subtilis*
Basal	2	468
Arabinose	2	1060
Xylose	350	500
Ribose	2	1880
Glucose	2	694
Fructose	4	757
Mannose	4	570
Galactose	5	450
Sodium galacturonate	1	400
Sucrose	4	680
Maltose	2	1900
Cellobiose	4	500
Sodium pectate	153	1090
Pectin	15	1490
Xylan	1170	627
Straw holocellulose	1060	652
Wheat straw	703	1020
Wheat bran	3850	2550
Wheat flour pentosan	2040	720
Oat hulls	364	1510
Beet molasses	66	1730
Sulphite waste liquor	408	3600
L.S.D. ($P = 0.05$)	110	195

Medium: 2% carbohydrate, 0.4% $(NH_4)_2HPO_4$, 0.1% KCl, 0.2% $CaCO_3$, 0.103% salt mixture, biotin, and 0.5% (v/v) ammonium lactate.

such as pectin, wheat straw, oat hulls, and beet molasses (Table II). Although wheat bran is superior to any of these, the greatest yields of enzyme have been obtained consistently with sulphite liquor. Since this culture is known to produce large amounts of amylase, which at the present is not considered a desirable attribute, the production of pentosanase on sulphite liquor was not investigated further. It is possible, however, that *B. subtilis* may produce little or no amylase when grown on sulphite liquor.

Effect of Bran Treatments

Most of the pentosan present in wheat bran exists in a water insoluble form and is not readily available to *B. pumilus*. Two possible methods of making the material more available are by grinding or by extracting with alkali. In addition, bran was treated briefly with hydrochloric acid to determine if bran hydrolyzate would provide a better substrate.

The bran was ground in a Wiley mill to pass screens of 20, 40, 60, 80, and 100 mesh. In each particular grind, particles smaller than the next lower mesh were removed by a screen classifier. A large increase in yield was obtained with bran ground to pass a 20 mesh screen as compared with unground bran (Table III). However, no further benefit was obtained by finer grinding.

TABLE III

EFFECT OF PARTICLE SIZE AND CHEMICAL TREATMENT OF BRAN ON YIELD OF PENTOSANASE

Treatment	Pentosanase, units/ml.	
	40 hr.	64 hr.
Control	780	1090
Particle size		
20 to 40 mesh	1000	1320
40 to 60 mesh	930	1250
60 to 80 mesh	970	1300
80 to 100 mesh	1040	1260
100 mesh	1020	1040
L.S.D. ($P = 0.05$)	90	95
Control	937	1140
NaOH under N_2	16	770
NaOH under air	6	750
HCl 20° C.	912	1180
HCl 115° C.	0	520

Medium: bran 2%, $(NH_4)_2HPO_4$ 0.4%, KCl 0.1%, $CaCO_3$ 0.2%, salts 0.103%, and biotin 2 γ per liter.

Considerable heat was produced in grinding bran to pass these smaller screens. This may be responsible for the lack of additional response with the finer brans since heat is known to decrease the solubility of hemicelluloses in plant material (11).

Extraction of bran with alkali was done in atmospheres of nitrogen and of air. One-gram lots of bran were weighed into 500 ml. Erlenmeyer flasks, 20 ml. of 0.1 *N* NaOH added, and the flasks shaken for 30 min. on the rotary shaker at 30° C. The acid hydrolyzates of bran were prepared by heating 1-gm. lots of bran with 20 ml. of 0.1 *N* HCl for 10 min. in an autoclave. They were then neutralized with 5 ml. of equivalent acid or base and 25 ml. of previously sterilized double strength medium aseptically added.

Both extraction of bran with alkali and hydrolysis with acid in the autoclave produced an inferior substrate for the production of enzyme (Table III). Since both treatments could destroy a number of growth factors present in the bran, the low yield may be a result of this loss in combination with the production of toxic substances such as furfural.

A Medium for Production of Pentosanase by B. pumilus

Selection of Ingredients

Wheat bran (2%), ground in a Wiley mill to pass a 20 mesh sieve, was selected as the carbohydrate, and corn steep liquor (1.23%) as the best nitrogenous adjunct to the basal medium for the production of pentosanase by *B. pumilus*. Studies were conducted to determine if any of the ingredients

in the basal medium could be omitted without reducing the yield. The addition of biotin to this medium, as was expected, did not have any effect on the production of enzyme and was therefore omitted. Each of the remaining ingredients, with the exception of bran, was withdrawn from the medium singly, and in groups of two, three, and four. Both ammonium phosphate and the trace salts could be omitted without a reduction in yield of enzyme. Potassium chloride was toxic in the presence of corn steep liquor, but in the absence of corn steep liquor, it was an essential constituent of the medium. Subsequently, it was found that when potassium chloride was replaced with sodium chloride higher yields were obtained either in the presence or absence of corn steep liquor. Calcium carbonate was required whenever ammonium phosphate was omitted from the medium, and this effect was intensified when corn steep liquor was simultaneously withdrawn. In the presence of corn steep liquor the effect of calcium carbonate was less marked. The final medium thus contained wheat bran, corn steep liquor, sodium chloride, and calcium carbonate.

Optimum Concentration of Ingredients

The optimum concentration of calcium carbonate and sodium chloride was determined in the presence of 1% corn steep liquor and 4% bran. The concentrations of both ingredients were varied simultaneously in a preliminary non-replicated factorial experiment. The optimum concentration of calcium carbonate was then checked in the presence of 0.05% NaCl, and that of sodium chloride in the presence of 0.1% $CaCO_3$. The average yields obtained from the assay of four flasks sampled at 40 and 64 hr. are depicted in Fig. 3. Both compounds have a higher optimum concentration in the earlier phase of the fermentation than in the later phase. With calcium carbonate, this is likely a reflection of better pH control during the first 24 hr. of the fermentation

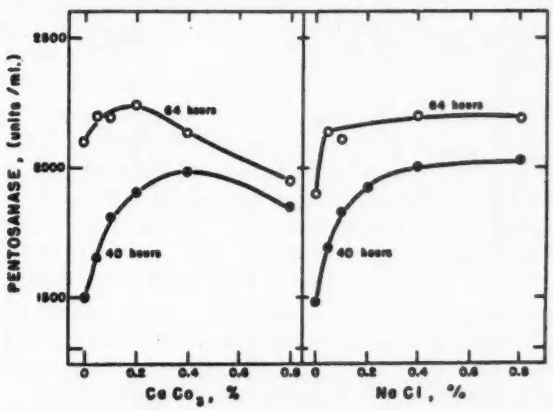

FIG. 3. Optimum concentration of calcium carbonate and sodium chloride in a medium containing 4% wheat bran and 1% corn steep liquor (30° C.).

when the organism is producing acids from the readily available carbohydrate material and using ammonium ion for nitrogen. The subsequent consumption of organic acids such as lactic and of protein carbon then causes the pH to rise so that at 40 hr. it is back to the original pH or higher. Thus, when the flasks are sampled at 40 hr. those flasks containing 0.4% $CaCO_3$ give the higher yields whereas by 64 hr. the effect of better pH control in the early part of the fermentation is less marked and 0.05 to 0.1% $CaCO_3$ appears sufficient. A concentration of 0.05% was selected as the optimum. A similar situation and one more difficult to understand existed with sodium chloride. When sampled at 64 hr. maximum yields were obtained with 0.05 to 0.1% NaCl independent of the concentration of calcium carbonate. A concentration of 0.05% was selected as the optimum.

The optimum concentration of corn steep liquor and bran was determined in the presence of 0.05% $CaCO_3$ and 0.05% NaCl. Again the corn steep liquor and bran were varied simultaneously in a preliminary non-replicated factorial experiment. The optimum concentration of corn steep liquor was then checked with media containing 6% bran and that of bran in media containing 1% corn steep liquor. The average yields of pentosanase obtained from four flasks sampled at 40 and 64 hr. are shown in Fig. 4. A concentration of 1% corn steep liquor and 6% bran was selected as the optimum level.

Production Curve

The medium, 6% bran, 1% corn steep liquor, 0.05% NaCl, and 0.05% $CaCO_3$, was used to determine the relationship between production of enzyme and time at 26° C. Four flasks were removed from the rotary shaker at intervals, the water lost by evaporation restored, and the pentosanase activity

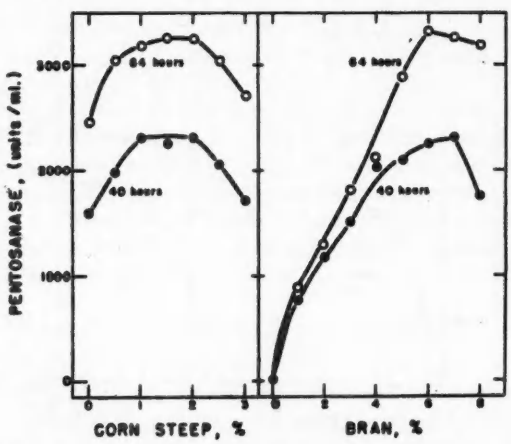

FIG. 4. Optimum concentration of corn steep liquor and bran in a medium containing 0.05% sodium chloride and 0.05% calcium carbonate (30° C.).

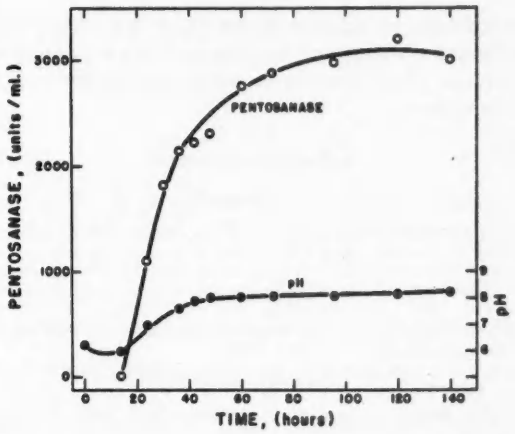

FIG. 5. Production of pentosanase at 26° C. by *B. pumilus* in a medium containing 6% wheat bran, 1% corn steep liquor, 0.05% sodium chloride, and 0.05% calcium carbonate.

and pH determined. The results are depicted in Fig. 5. After inoculation there is a lag of five to seven hours before a measurable amount of oxygen is consumed.* The amount of enzyme in the medium rises quickly between 12 and 40 hr. and then gradually increases until the maximum is reached at approximately 96 hr. The pH drops slightly from 6.4 to 6.0 and then rises to 8.0 within 48 hr. The enzyme, as will be shown in a following paper, is quite stable up to pH 11.0. Growth in 24 hr. was very dense and by 40 hr. the majority of the cells had sporulated. It is quite probable that if the pentosan material in the bran could be made more readily available to the organism without producing toxic substances or destroying growth factors that the maximum yield of pentosanase would be reached at this time. The rate of enzyme production would then likely be similar to that previously obtained in the basal medium with xylose and yeast extract.

The yields of pentosanase obtained with this commercial production medium are of the same order as those initially obtained with xylose, yeast extract, mineral salts, and biotin. The bran medium has been used to produce enzyme in 30-liter stainless steel fermentors with yields similar to those obtained in shake flasks. The bran, however, in this scale was sometimes difficult to sterilize. Mixing and heating a thick slurry of the bran before adding to the fermentors and sterilizing was found helpful.

Although *B. pumilus* does not secrete amylase it does secrete proteolytic enzymes, and the culture liquors from the bran medium exhibit strong proteolytic activity as well as pentosanase activity. *B. subtilis* does produce amylase and protease, but the good yields of pentosanase obtained from this

* *Kindly determined by Dr. Ping Shu in his apparatus for measuring rate of oxygen uptake (6).*

organism when cultured on sulphite liquor make it an attractive organism for production purposes. A number of molds are known to secrete pentosanases and in a subsequent paper a study of some factors affecting production by two fungi will be reported.

Acknowledgments

The author wishes to express his appreciation to Dr. Ping Shu for determining the rate of oxygen uptake and to Mr. A. S. Sieben for his technical assistance.

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THE APPLICATION OF BACTERIOPHAGES TO THE STUDY OF ACID-FAST MICROORGANISMS FROM TUBERCULOUS PATIENTS[1]

By Stephen I. Hnatko[2]

Abstract

The bacteriophage pattern of 24 classified acid-fast microorganisms is presented and compared to that of a group of unclassified mycobacteria isolated from tuberculous patients. Of 33 unclassified acid-fast cultures studied 24 (73%) were typed satisfactorily; these comprised 93% of the cultures isolated from gastric lavage, 75% of those isolated from urine, and 29% from sputum. The majority of these fell into a *Mycobacterium smegmatis* phage pattern.

Introduction

The widespread distribution of acid-fast bacilli and the difficulty of differentiating pathogenic from saprophytic organisms by morphological and cultural appearances suggest that bacteriophages may become invaluable in the classification of strains within the genus *Mycobacterium*. Phage-typing of strains within a bacterial species has proved significant in epidemiological studies of typhoid (1, 3, 4, 12), enteric infections (2, 7), staphylococcal infections (11, 8), and less so in the investigations of *Pseudomonas* (5, 18), *Corynebacterium* (6, 17), and *Mycobacterium* (13, 14, 15, 16, 19). The application of phages to the classification of acid-fast bacteria has been little investigated since the first discovery of a phage against *M. smegmatis* (10, 14, 16). The stability of "mycobacteriophages" and of the phage patterns shown by certain species within the mycobacterium genus suggests that phages may be applicable to the typing of acid-fast microorganisms (13, 14, 15, 16). The practical value of such a procedure in clinical tuberculosis will have to await assessment.

The present paper deals with the application of "mycobacteriophages" to the classification of non-pathogenic acid-fast organisms isolated from tuberculous patients. The term "non-pathogenic" means that cultures were not pathogenic for guinea pigs in the usual doses. The clinical value of such a study is discussed.

Materials and Methods

Media

The media used were 2% glycerol nutrient broth (13) and T.B. broth base with Tween 80 (Difco) modified by the substitution of 5% liquoid (Roche)-treated sheep serum for bovine plasma fraction V. Briefly the sheep serum

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TABLE I

HISTORY OF SAPROPHYTIC ACID-FAST BACILLI ISOLATED FROM TUBERCULOUS PATIENTS

Hospital diagnosis	Strains isolated*	Source of strains	Patients from whom both saprophytic and pathogenic organisms isolated and source**
Pul. tbc. mod. adv.	30776, 31002, 28828, 31003, 30777 3188, 30778, 8016, 1367, 1824 13243, 12369, 5917	Gastric lavage (G.L.)	30776(G.L.), 28828(G.L., sputum) 31003(G.L., sputum), 30777 (G.L., sputum), 1824 (G.L.)
Tbc. pneumonia	31497	Gastric lavage	
Pul. tbc. far adv.	4923, 26119	Sputum	4923 (chest fluid, sputum)
Renal tbc. pul. tbc. mod. adv.—non-bacillary	2362	Urine	
Perinephric abscess	3210	Urine	
Tbc. appendicitis, enteritis	5183	Urine	
Pleural effusion, renal tbc.	3158	Urine	Sputum
Tbc. cystitis—quietly active	3147	Urine	Sputum
Left pleural effusion pul. tbc.—primary active	1193	Urine	
Prim. pul. tbc. non-bacillary	5184	Laryngeal swab	
Pul. tbc. suspected. Ca. of lung—suspected	8424	Bronchial secretion	

* *Each strain no. refers to an organism isolated from a different patient.*

** *Patients from whom both tubercle bacilli and saprophytic organisms were isolated, e.g. 30776(G.L.) means that from this patient a saprophytic strain 30776 was isolated in addition to a pathogenic tubercle bacilli from gastric lavage.*

was treated by the addition of 1 mgm. liquoid plus 1 ml. 1/1000 $CaCl_2$ per 1 ml. sheep serum. The mixture was allowed to stand at room temperature for 20 min. It was centrifuged at 3000 r.p.m. for 20 min. and the resultant supernatant was Seitz sterilized. The sterile supernatant was added to T.B. broth media aseptically in proper concentration. The value of liquoid-treated sheep serum as a growth factor for *M. tuberculosis* was studied over a period of one year (results will be published separately). All media were solidified with agar (Bacto) prior to autoclaving.

Cultures

The cultures studied were acid-fast strains recently isolated from patients who were admitted to sanatoria for investigation or for the treatment of tuberculosis. None of the saprophytic strains were pathogenic to guinea pigs. The source of these strains and the clinical and bacteriological assessment of the patients from whom they were derived is shown in Table I.

Bacteriophages

The bacteriophages used consisted of the following types: (1) *M. smegmatis* (MS_1-MS_9), (2) *M. phlei* (MT_1-MT_4), (3) *M. lacticola* (ML_1), (4) *M. rabinowitschi* (MRa), (5) *M. pellegrino* (MP_1), (6) *M. eos* (ME_1), (7) *M. ranae* (MR_1), (8) MT_2L/Ph_2 and (9) D_{29}. Their sources and properties have been previously reported (13, 14, 15, 16). Both stock phage (undiluted) and 10^{-3} dilution were used. Phage D_{29} was received through the courtesy of Dr. S. Froman, Olive View Sanatorium, California. This phage was stated to be active against *M. tuberculosis* H_{37Rv} (9), but such activity has not been too convincingly demonstrable in the present investigation. The phage has the host pattern of an *M. smegmatis* type.

Procedure for Phage-typing of Acid-fast Microorganisms

Nutrient agar and liquoid modified T.B. agar plates were flooded uniformly using a Pasteur pipette and a 48–72 hr. broth culture, dried, and spotted with appropriate phage (15). Spotting consisted of placing with a Pasteur pipette a small drop of a phage suspension on a marked area. With suitable identification marks as many as 20 phages could be tested on one plate. The plates were dried and incubated at 37° C. and read for lysis (area of clearing) of culture at 24 hr. with final reading at 48 hr. An area of lysis of about four milliliters was considered as confluent lysis. The phage pattern obtained against the unknown strains was compared to the phage pattern of a classified group of organisms. In that manner the unclassified strains were classified as to possible species type. These strains were tested with the bacteriophages periodically over two years.

Results

Table II shows the phage pattern of a group of known acid-fast bacilli.

Of six strains of *M. phlei* obtained from different centers five belonged to the same phage pattern. On the basis of phage activity these strains are most probably alike. One strain, *M. phlei* 502, was unclassifiable. Of *M. smegmatis*

strains tested four different types were found, but strains of the avirulent *M. tuberculosis* ATCC 607 obtained from three centers showed the same phage pattern. This suggests that strains can be maintained in different laboratories without change or variability in their phage sensitivity. This does not conflict with the fact that the phage pattern presented here differs slightly from those reported previously (15), owing to certain alterations in the phages used. MS_2 phage previously active for *M. phlei* strains was found to contain two different phage types, and some inevitable variation in the titers of phages employed has led to slight overlapping of patterns.

Of 33 unclassified acid-fast strains studied 24 (73%) were typed by one or more of the phages used (Table III). These comprised 93% of cultures isolated from gastric lavage, 75% of those isolated from urine, and 29% from sputum. The majority of these fell into a *M. smegmatis* phage pattern.

Strains 30776 and 31002, though isolated from the same source and from the same patient, revealed different phage patterns whereas strains 3147 and 3158 from different sources both belong to the same phage pattern.

None of the strains tested were acted upon by the *M. phlei* phages. This may indicate the infrequent occurrence of this organism in the human body.

TABLE II

BACTERIOPHAGE ACTIVITY PATTERN OF CLASSIFIED ACID-FAST ORGANISMS

Strain tested and source	Bacteriophage pattern	Species and type**
M. phlei (Toronto)	MS_1-MS_9 ML_1 MR_9 MR_1 D_{29} MT_1-MT_4 MT_2L/Ph2	A
M. phlei 178 (Ottawa)	MT_1 MT_4 MT_2L/Ph2	B
M. phlei ATCC 355 (Ottawa)	MT_1 MT_4 MT_2L/Ph2	
M. phlei (Edmonton)	MT_1 MT_4 MT_2L/Ph2	
M. phlei (Rome)	MT_1 MT_4 MT_2L/Ph2	
M. phlei ATCC 10192 (Hamilton)	MT_1 MT_4 MT_2L/Ph2	
M. phlei 502 (Ottawa)		
M. smegmatis (Toronto)	MS_1-MS_8 MS_7-MS_9 ML_1 D_{29}	A
M. smegmatis (Washington)	MS_1-MS_8 MS_7-MS_9 ML_1 D_{29}	
M. smegmatis (Rome)	MS_1-MS_8 MS_7-MS_9 ML_1	B
M. smegmatis (Toronto)	MS_1-MS_8 MS_7-MS_9	C
M. smegmatis NRRL B-612 (Regina)	MS_1-MS_4 MS_6-MS_9 ML_1	D
M. eos 500 (Ottawa)	ME_1	A
M. lacticola 501 (Ottawa)	MS_1-MS_8 MS_7-MS_9 ML_1	A
M. fribergensis (Rome)	MS_1-MS_9 ML_1 MRa MR_1	A
M. rabinowitschi (Rome)	MS_1-MS_8 ML_1 MRa	A
M. pellegrino (Rome)	MP_1	A
M. ranae 503 ATCC 110 (Ottawa)	MS_1-MS_9 MR_1 MT_7-MT_8	A
Avirulent *M. tuberculosis* ATCC 607 (Toronto)		
No. 546 ATCC 607 (Ottawa)	MS_1-MS_9 ML_1 MRa MR_1 D_{29}	A
No. 71 ATCC 607 (California)	MT_7-MT_8	
M. tuberculosis var. *bovis* (?) (Edmonton)	MR_1/B*	

* MR_1/B = *M. ranae phage adapted to above strain. The strain is non-pathogenic for guinea pigs, fowl, or rabbits.*

** *This column refers to species and type according to phage pattern "A", "B", etc. to designate different types (strains) within the specified species.*

TABLE III

PHAGE PATTERN OF 24 UNCLASSIFIED ACID-FAST BACILLI

Strain No.	Phage pattern	Species and type*	
30776	MS_1 MS_2 D_{29}	*M. smegmatis*	
28828	" " "	*M. smegmatis*	(A)
3210	" " "	*M. smegmatis*	
31002	MS_1 MS_2 MS_5 ML_1 D_{29}	*M. smegmatis*	
31003	Same	*M. smegmatis*	
30777	Same	*M. smegmatis*	(B)
2424	Same	*M. smegmatis*	
31497	MS_1 MS_2 ML_1 D_{29}	*M. smegmatis*	(C)
3188	MS_4 D_{29}	*M. smegmatis*	(D)
30778	MS_1 MS_2 MS_5 ML_1	*M. smegmatis*	(E)
8016	MS_1 MS_2 MS_5 MS_6	*M. smegmatis*	
1367	Same	*M. smegmatis*	(F)
1824	MS_4 MS_6	*M. smegmatis*	(G)
13243	MS_4 MS_7	*M. smegmatis*	(H)
12369	MS_1 MS_2 MS_4 MS_7	*M. smegmatis*	(I)
5917	MS_7	*M. smegmatis*	(J)
4923	MS_1 MS_2 MS_7 ML_1	*M. smegmatis*	(K)
26119	D_{29}	*M. smegmatis*	
5183	D_{29}	*M. smegmatis*	(L) (?)
5184	D_{29}	*M. smegmatis*	
2362	MR_1	*M. ranae*	(A)
3158	ME_1	*M. eos*	
3147	"	*M. eos*	(A)
1193	MS_1 MS_2	*M. smegmatis*	(M)

* *This column according to phage pattern activity represents different types of microorganisms within the species given. These are simply designated types "A", "B", etc. and are not necessarily identical with types given in Table II.*

Discussion

The fact the "mycobacteriophages" have been shown to store well at refrigerator temperature (6° C.) and maintain their potency is evidence of their stability (13, 14, 15). The phage patterns of classified acid-fast strains studied in the last two years have not altered to any great extent. This is a factor in favor of their use in studying acid-fast bacteria. The methods used here are similar to those employed in the classification of staphylococci.

Of 33 saprophytic strains tested in this study 24 (73%) were lysed by one or more of the phages. Of these 21 fell into a *M. smegmatis* phage pattern. Ninety-three per cent of the strains isolated from gastric lavage were typed, 75% of those isolated from urine, and 29% from sputum. The investigation illustrates the widespread distribution of non-pathogenic acid-fast strains within the human body. It does not bear out the belief that *M. smegmatis* is associated only with the genitourinary tract. The organisms typed from urine were found to belong to at least three different species types, whereas those typed from gastric lavage were found to belong to the *M. smegmatis* type. *M. smegmatis* appears to be associated more so with upper intestinal tract.

The widespread distribution of saprophytic acid-fast bacilli and the demonstration of such organisms in sputum and gastric lavage of persons with demonstrable pulmonary disease and with no pulmonary disease is sufficient indication for careful investigation of the isolated microorganisms. This may be achieved by the use of bacteriophage.

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AN AGAR DIFFUSION ASSAY METHOD FOR THE ANTIFUNGAL POLYENE, CANDIDIN[1]

By Willard A. Taber[2]

Abstract

An agar diffusion cup assay method for candidin is described. Some conditions of the assay medium affecting growth rate of the test organism and diffusion rate of candidin are presented. Critical steps of the procedure were tested statistically for significant variation and found acceptable. Both the sodium salt and Zwitterionic forms of candidin can be assayed.

Introduction

Candidin is an antifungal substance (9) possessing a polyene chromophore (6). A single fraction is now recognized (11). This fraction consists of a single active substance (as demonstrated by paper chromatography (11)), which is water soluble when prepared as the sodium salt.

The heptaene series of polyenes (ascosin, candicidin, candidin, and trichomycin, see (6) for references) obtained from species of *Streptomyces* are difficult to work with (8) because of their stability and solubility properties, and none has been reported pure to date. An agar diffusion paper disk assay method has been described for candidin (9) but it was not completely satisfactory. A convenient method, reliable and flexible, was needed for further purification studies of candidin. Certain steps of the assay procedure have been varied in this investigation in an attempt to develop a more suitable procedure. Factors considered were: (*a*) sensitivity of the method, (*b*) length of incubation period required, (*c*) validity of the design of the assay previously used, and (*d*) reproducibility of the method. Candidin was available in three forms: a completely water soluble sodium salt, a neutral preparation consisting partly of sodium salt and partly of Zwitterion which dispersed to a cloudy solution in water (represented by the master standard), and the water insoluble Zwitterion (10). A means of assaying Zwitterionic samples against the water soluble standard was sought.

Some factors affecting the assay are described below, followed by a description of the assay procedure.

Factors Affecting the Assay

The method of applying the solutions to the agar plates was the first step of the existing method examined in an effort to increase sensitivity of the assay. The cylinder cup method was compared with the paper disk method and the former was found to produce larger zones for a given concentration. The cylinder cup method was adopted.

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Contribution from the National Research Council, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as Paper No. 212 on the Uses of Plant Products and as N.R.C. No. 3846.
[2]*A portion of this investigation was completed while the author was a Postdoctorate Fellow at the Institute of Microbiology, Rutgers University, the State University of New Jersey, New Brunswick, New Jersey, U.S.A.*

Composition of the Medium

The effects of varying certain constituents of the medium on diffusion rate of candidin and growth rate of the test organism, *Candida albicans* ATCC 10231, were examined in an effort to increase the sensitivity of the method and to decrease the incubation period required by the assay. It was considered more advantageous to select conditions which would increase sensitivity (increase diffusion) rather than decrease incubation time (increase growth rate) should a given condition not favorably affect both factors.

Each modification of the medium was prepared first without agar so that the effect of a modification on growth rate could be measured turbidimetrically. This was carried out by dispensing 4.9 ml. of sterile medium per sterile, dry colorimeter tube, inoculating with 0.1 ml. of washed cells (%T 10–15) and reading turbidity at 460 mμ using a Bausch and Lomb spectronic "20" colorimeter. The data represented in Figs. 1 through 5 are 36 hr. readings. The above wave length was selected according to the criteria set forth in (4). The remainder of the modified medium in each case was then mixed with the appropriate amount of sterile, dry agar, melted in a water bath, and dispensed in metal top Petri dishes. The effect of the modification on diffusion rate was measured, in terms of zone diameter, following application of candidin to metal cylinders placed on the agar plates. The candidin standard used throughout this investigation was a sodium salt having 6136 units/mgm. activity.

The Effect of Glucose

An increase in glucose concentration from 2 to 15 gm. per liter increased growth rate whereas an increase from 2 to 50 gm. did not affect diffusion rate of candidin (Fig. 1). An excess of glucose, 30 gm./liter, was selected for the assay medium.

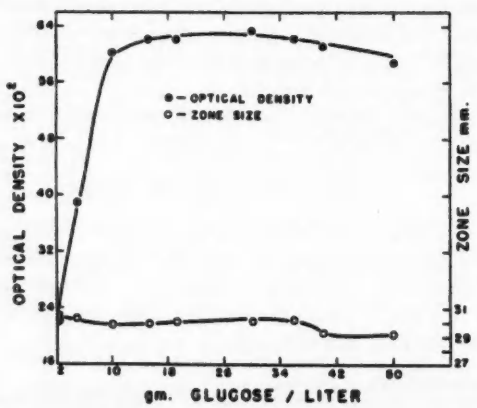

FIG. 1. Effect of varying the glucose concentration of the assay medium on the growth of the test organism, *Candida albicans*, and on diffusion of candidin. Candidin was applied by the cup method at a concentration of 10 µgm./ml. after addition of agar to the media.

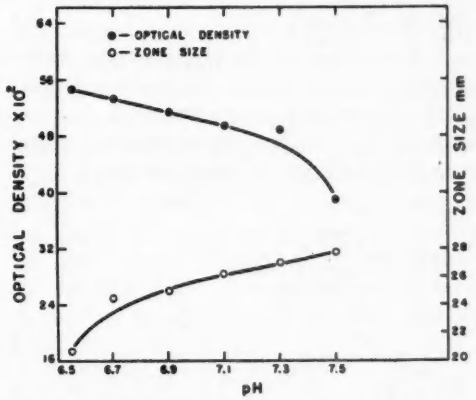

FIG. 2. Effect of varying pH of the assay medium on growth of the test organism, *Candida albicans*, and on diffusion of candidin. Regulation of pH was with *M*/15 phosphate buffer. Candidin was applied by the cup method at a concentration of 10 μgm./ml. after addition of agar to the media.

The Effect of pH

The effect of the pH of the medium on growth rate and diffusion rate was determined by replacing the dibasic potassium phosphate of the original medium with varying proportions of Sorensen's phosphate buffer solutions to give a final concentration of *M*/15 (Fig. 2). A high pH was selected for the medium. Since phosphate inhibited diffusion (see below), the pH was raised by adjustment of the medium prior to autoclaving, to pH 8.1 with 40% NaOH. The reaction after melting once ranged from pH 7.4 to 7.6.

The Effect of Phosphate

As a part of the above study, the effect of varying the concentration of Sorensen's phosphate buffer was examined. The proportions of solutions to give a reaction of pH 7 were used. An increase in the concentration of phosphate salt to 24 mM. increased growth rate and decreased diffusion rate (Fig. 3). Dibasic potassium phosphate was selected as the most efficient phosphate source for the medium. Concentrations varying from 100 to 750 mgm./liter were examined. Growth of *Candida albicans* and diffusion of candidin were affected as when using Sorensen's phosphate buffer (data not included). A concentration of 150 mgm./liter was selected.

The Effect of Yeast Extract

Yeast extract was added as a vitamin source (5). Increasing the yeast extract concentration within the limits examined caused an increase in growth rate and a corresponding decrease in diffusion rate (Fig. 4). A concentration of 45 mgm./liter was selected to effect a compromise between the two responses.

The Effect of Sodium Chloride

Sodium chloride had been used in the original assay medium. Varying the concentration of sodium chloride within the limits examined had no effect on growth rate but did increase diffusion rate (Fig. 5). Commensurate with increased zone diameter, however, was a progressive loss in margin sharpness. Sodium chloride was omitted from the assay medium.

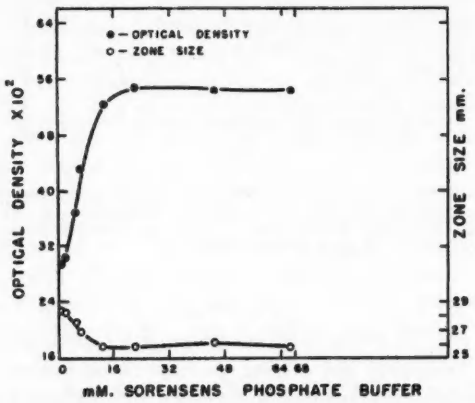

FIG. 3. Effect of varying the concentration of phosphate (as Sorensen's buffer system, pH 7) of the assay medium on growth of the test organism, *Candida albicans*, and on diffusion of candidin. Candidin was applied by the cup method at a concentration of 10 μgm./ml. after addition of agar to the media.

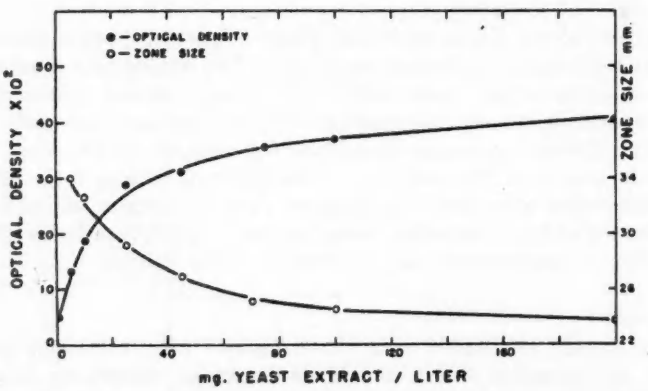

FIG. 4. Effect of varying the yeast extract concentration of the assay medium on growth of the test organism, *Candida albicans*, and on diffusion of candidin. Candidin was applied by the cup method at a concentration of 10 μgm./ml. after addition of agar to the media.

Diluent

The use of sodium carbonate as a diluent gave the most extensive dose–response range. However, although the sodium salt of candidin was readily soluble in this solvent, the master standard and Zwitterionic preparations were not. The master standard was soluble in dilute sodium hydroxide, pH 10, and once dissolved could be diluted into sodium carbonate (50 mgm./liter, pH 9.8). This system appeared promising provided that the sodium hydroxide stock solution of candidin was sufficiently stable. Five milligrams of the standard was dissolved in sodium hydroxide and at intervals portions were diluted into sodium carbonate solution to a concentration of 10 μgm./ml. and applied to agar plates (Fig. 6). It was concluded that breakdown during the time normally required to prepare the dilutions from the stock solution would not be significant.

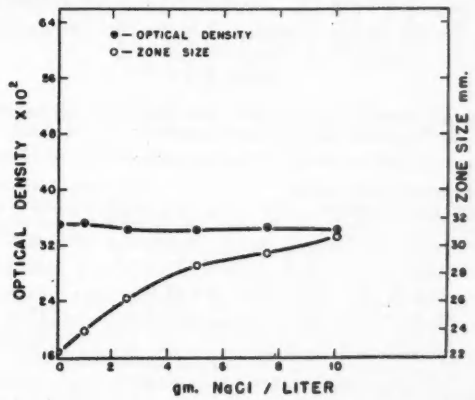

FIG. 5. Effect of varying the sodium chloride concentration of the assay medium on growth of the test organism, *Candida albicans*, and on diffusion of candidin. Candidin was applied by the cup method at a concentration of 10 μgm./ml. after addition of agar to the media.

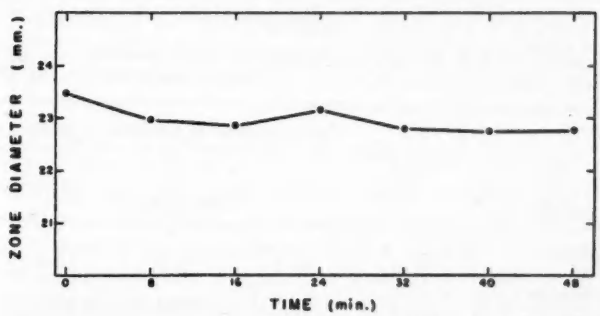

FIG. 6. Zone diameters produced by candidin stock solution (pH 10) following standing for various periods of time at room temperature. At intervals, portions of the stock solution (500 mgm./ml.) were diluted to 10 μgm./ml. with Na_2CO_3 solution and applied to agar plates.

The stability of the sodium carbonate dilutions prepared from the stock solution was examined by preparing complete dose–response curves at initial, 15 min., and 30 min. intervals. Breakdown following standing for 15 min. was not significant (see Table I for data of one concentration). Breakdown following 30 min. standing was questionable but probably significant. It was apparent that while breakdown of the stock solution and sodium carbonate dilutions was not significant over short periods of time, breakdown probably did occur during the time required for the margins of inhibition to be set. Careful control of time should standardize the rate of breakdown between the standard and unknown, should it in fact occur. Simultaneous preparation of the sample to be assayed and the standard permitted satisfactory recovery of activity (Table IV).

The Zwitterionic form of candidin was not soluble in sodium hydroxide at pH 10 but was soluble at pH 11. Sodium hydroxide at pH 11 could be used for preparation of the stock solutions if both the Zwitterion and standard,

TABLE I

A COMPARISON OF ACTIVITY, EXPRESSED AS ZONE DIAMETER, OF AN ALKALINE SOLUTION OF CANDIDIN BEFORE AND AFTER STANDING 15 MIN.

Zone diameter, mm., produced by 5 μgm./ml. concentration			Zone diameters produced by same solution 15 min. later		
18.5	18.5	17.8	18.3	18.1	18.7
18.7	18.5	17.6	17.2	18.0	17.9
18.9	18.4	17.4	17.0	16.9	17.5
17.6	18.0	18.2	17.5	18.4	18.1
Mean	18.18			17.80	

Difference of means, 0.38.
Standard error of difference of means, 0.217.
$t = \frac{0.38}{0.217} = 1.75.$
Tabulated t value for 22 degrees freedom at the 5% level, 2.07.

TABLE II

A COMPARISON OF THE ACTIVITY, EXPRESSED AS ZONE DIAMETER, OF THE CANDIDIN STANDARD WHEN DISSOLVED IN SODIUM HYDROXIDE SOLUTIONS AT pH 10 AND 11

	Concentrations of solutions, μgm./ml.				
	20	15	10	7	5
Treatment	Zone diameter				
Sodium hydroxide pH 10	22.3	21.2	18.5	16.4	14.0
Sodium hydroxide pH 11	22.3	21.0	18.1	16.2	13.9
Difference of means		0.2	0.4	0.2	0.1
t		0.65	0.85	0.49	0.22
d.f.		30.0	26.0	30.0	28.0

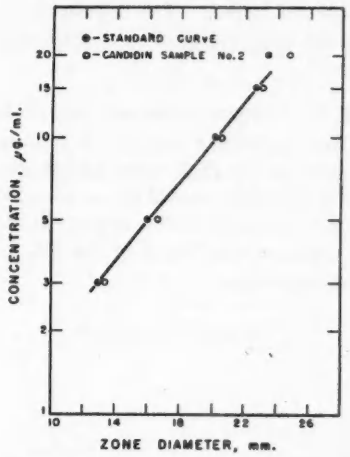

FIG. 7. Dose–response curves of the candidin standard and of candidin lot No. 2 (Zwitterion form). Both were dissolved in sodium hydroxide, pH 11, and diluted into sodium carbonate solution.

dissolved at pH 11, reacted similarly. The effect on diffusion rate of small variations in pH of the stock solutions of the neutralized standard and Zwitterion form, when dissolved in sodium hydroxide of pH 11, was examined indirectly by dissolving portions of the standard at pH 10 and 11 (Table II). No significant difference existed between the two preparations. The dose–response curves produced by the standard and Zwitterion form dissolved at pH 11, then diluted into sodium carbonate, were parallel (Fig. 7). Tests similar to those described in the two previous paragraphs, but using sodium hydroxide of pH 11, indicated that this initial solvent was satisfactory. In addition, various dilutions of the standard were assayed against the standard (using pH 11 initial solvent) and theoretical and calculated potencies were compared. The stock solutions and their dilutions were prepared simultaneously. Calculated potency agreed within $\pm 5\%$ of theoretical potency in three separate assays.

Preparation of Agar Plates

A comparison was made of the single and double layer agar techniques. Although the dose–response curve was little affected, slightly sharper zones at the lowest level of candidin were obtained when using the double layer technique. The latter was adopted for use in the assay.

Concentration of the Inoculum

In order to determine whether the dose–response relationship was sensitive to the size of the inoculum, equal amounts of agar were seeded with cell suspensions differing sixfold in concentration (by dilution). A slight

difference in curve slope was noted. The heavier inoculum was used for the assay method because the zone margins were more distinct at the end of the 36 hr. incubation period.

Pattern of Distributing the Concentrations over the Plates

Tests were made for significant variations in zone diameter when four samples of one concentration per plate were added and a replica of four were used. This pattern, if acceptable, would be convenient for purification studies because of its flexibility. Variation either among plates of a replica or among the total of the zones was not significant at the 5% level (Table III). This pattern was considered satisfactory.

Assay Procedure

Assay Medium

Stock solution Y	
Yeast extract	450 mgm.
Distilled water	to 10 ml.
Solution A	
Anhydrous glucose	30 gm.
Distilled water	200 ml.
Solution B	
$(NH_4)_2SO_4$	1.5 gm.
K_2HPO_4	150 mgm.
$MgSO_4 \cdot 7H_2O$	100 mgm.
$ZnSO_4 \cdot 7H_2O$	0.01 mgm.
$FeSO_4 \cdot 7H_2O$	0.01 mgm.
Distilled water	800 ml.
Adjust to pH 8.1 with 40% NaOH agar	15 gm.

Each solution is autoclaved for nine minutes at 16–18 lb./sq. in. Solutions A and B and 1 ml. of stock solution Y are mixed and dispensed in *ca.* 200 ml. amounts per sterile, dry 250 ml. Erlenmeyer flask. This procedure avoids reheating of any lot of agar since only the required amount is melted in a water bath for a given assay.

Inoculum

Candida albicans ATCC 10231 is maintained on an agar medium of the following composition: glucose 10 gm.; Bacto peptone 5 gm.; beef extract 5 gm.; sodium chloride 5 gm.; agar 15 gm.; and distilled water 1 liter (12). The inoculum is prepared by suspending a 48 hr. slant grown at 28° in 7 ml. water. This is adjusted to a per cent transmission of 5–10. Seven milliliters are used per 400 ml. agar.

Preparation of the Plates

The required number of flasks of agar are placed in a boiling water bath and heated just long enough to melt the agar. The agar is pooled in one large

TABLE III

ANALYSIS OF THE VARIATION EXISTING AMONG PLATES (COLUMNS) AND ZONES (ROWS) WITHIN A REPLICA OF FOUR PLATES

3 μgm./ml. concentration

	Plates				Total
	(1)	(2)	(3)	(4)	
Zones	16.0	17.1	15.8	15.6	— 64.5
	16.0	15.8	16.7	15.6	— 64.1
	16.6	16.3	16.3	16.1	— 65.3
	15.9	15.2	15.3	15.7	— 62.1
Total	64.5	64.4	64.1	63.0	

Analysis of Variance

Variation	Degrees freedom	Sum of squares	Mean square	*F*
Total	15	3.88	0.258	
Plate	3	0.35	0.116	2.05
Zone	3	1.39	0.463	1.94
Interaction	9	2.14	0.238	

Note: $n_1 = 3$, $n_2 = 9$.

dry flask and after gentle mixing, 10 ml. are added per plate. The remainder of the agar is cooled 20 min. in a 47° C. water bath and then seeded. Four milliliters of seeded agar are added per plate, and after being chilled for one hour, the metal cylinders are placed in position. The prepared plates which are used the same day are kept at 4° C. until the dilutions are applied to the cups. The cups are uniformly filled with a fine dropper.

Preparation of the Dilutions

Five milligrams of candidin are dissolved in either sodium hydroxide pH 10 or pH 11, depending upon whether the sample is the sodium salt or the Zwitterionic form. Dilutions of 15, 10, 7, 5, and 3 μgm./ml. are prepared by diluting into sodium carbonate solution (50 mgm./liter, pH 9.8). The dilutions of the standard and unknown are prepared simultaneously to reduce difference in breakdown due to differences in time of standing.

Incubation

It was not found feasible to reduce the incubation period by altering conditions of the assay to favor growth rate of the test organism. The plates are incubated for 36, or preferably 48 hr. at 37° C.

Measurement of Zones

Zone diameters are measured to the nearest tenth of a millimeter using a Fisher–Lilly zone reader.

Test for Validity of the Assay

Dose–response curves for the standard and unknown are plotted on semilogarithmic paper. If the curves appear parallel by visual inspection the analysis is continued. The degree of agreement between the two curves is tested by comparing potencies estimated for several different concentrations. Relative potency is computed by the method described below for the following concentrations: (*a*) 15 and 10 μgm./ml., (*b*) 15 and 5 μgm./ml., (*c*) 10 and 7 μgm./ml., and (*d*) 10 and 5 μgm./ml. These estimates are tested for homogeneity using the goodness of fit test (7) ($\text{Chi}^2 = \Sigma (\bar{x} - X)^2/\bar{x}$, where $\bar{x}$ and X represent the average and the individual values respectively). The assay is considered valid if the individual estimates do not differ significantly at the 5% level.

Estimation of Relative Potency

Relative potency is determined using the expression of de Beer and Sherwood (1).

$$\text{Relative potency (\%)} = \text{antilog}\left[2 + d\left(\frac{(U_2 + U_1) - (S_2 + S_1)}{(U_2 - U_1) + (S_2 - S_1)}\right)\right]$$

where d is the log ratio of the higher concentration to the lower concentration, and S and U refer to the sum of the zone diameters for the two indicated concentrations of the standard and unknown.

The potencies estimated using four concentrations are averaged and the 5% fiducial limit (7) based on the variation among the estimates is computed. The potency of a sample is recorded as this average and the 5% fiducial limits (Table IV).

The above methods of testing validity of an assay and of pooling the data of several concentrations within a single assay are not standard procedures such as those recommended by (2) and (3). The procedure described above, however, does provide a rapid method of computation which is suitable for purification studies, and has been found superior in reproducibility to the

TABLE IV

ESTIMATION OF POTENCY OF A SAMPLE OF THE STANDARD PREPARATION HAVING 5030 UNITS/MGM. ACTIVITY*

Concentration points used, μgm./ml.	Estimated potencies	Chi²	Standard error, $S_{\bar{x}}$	"Fiducial limit" $\pm t(05)S_{\bar{x}}$	Estimation of potency
15 and 10	4779				
15 and 5	4888				
10 and 7	4872	2.4	31.0	98.58	4827 ± 98.58
10 and 5	4768				
$\bar{x}$	4827				

* *The sample was assayed against the standard as though identical dilutions were used. It actually contained 18% less candidin by weight.*

graphical method. Samples recovered from purification studies ranging from 20% less active than the standard to about 100% more active than the standard have been satisfactorily assayed by this method.

Examination of candicidin, trichomycin, and culture filtrates of fresh isolates of *Streptomyces* shown, by ultraviolet absorption spectrum analysis of butanolic extracts, to produce polyenes, suggests that this assay method might have application for polyenes other than candidin. A number of polyenes have been reported from *Streptomyces* (see (11) for a partial list) and it has been the experience of the author that they are frequently encountered in antifungal screening programs. This method has been described in detail in the event that it might prove useful to those concerned with the isolation and assay of polyene substances.

Acknowledgments

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MICROBIOLOGICAL UTILIZATION OF CELLULOSE AND WOOD

I. LABORATORY FERMENTATIONS OF CELLULOSE BY RUMEN ORGANISMS[1]

By D. W. Stranks

Abstract

Fermentations of cellulose were carried out using rumen microflora. Butyric, propionic, acetic, and traces of formic acids were produced from the cellulose fermented. When peptone was present and the pH of the mash was maintained at 6.5 and the end products of fermentation were removed continuously by a dialysis ion exchange system, 84% of a 4.12% cellulose suspension was fermented in 72 hr. at 40° C. Fermentations using rumen organisms might become a means of utilization for low forms of wood waste.

Introduction

In the search for microbiological agents that might be utilized to ferment wood waste, attention was drawn to the rumen microflora. These organisms are known to decompose cellulose at a rapid rate *in vivo*, are seemingly not inhibited by the presence of lignin in feedstuffs such as grass, hay, and straw, and are capable of producing short-chain fatty acids and other substances from the substrate attacked.

In nature these rumen organisms live in a symbiotic relationship with the host animal and are so well nurtured by it that removal from their natural milieu presents difficulties in handling not encountered with most organisms. In order to facilitate study of these organisms and to simulate the required conditions of their natural habitat as nearly as possible, initial studies were carried out using large volumes of rumen contents, i.e., *in vitro* fermentations. Such investigations, it was assumed, would give some idea of the general suitability of these organisms for the fermentation of cellulose and wood.

Sheep rumen fermentations have been carried out *in vitro* by Marston (3) using a special fermentation vessel. Sodium bicarbonate was added when necessary to control the pH of the fermenting mash. Butyric, acetic, and propionic acids and methane and carbon dioxide were produced from the cellulose decomposed. Working with approximately 2% cellulose suspensions, he was able to decompose 33–68% of the cellulose present. Louw *et al.* (2) using dialysis to remove the end products of fermentation and manual pH control were able to decompose 16–36% more cellulose (initial cellulose suspensions were 2–3%) than was possible when end products were not removed. On the other hand, Hungate (1), discussing the feasibility of industrial fermentations of cellulosic substances, considered the concentration of cellulose suspensions that were fermented by bacteria in general too low for practical fermentations. He mentioned McBee's unsuccessful attempt (unpublished) to remove inhibiting substances by solvent extraction as a

[1]*Manuscript received in original form April 25, 1955, and as revised, November 3, 1955.*
Contribution from the Ottawa Laboratory, Forest Products Laboratories of Canada, Division of the Forestry Branch, Department of Northern Affairs and National Resources.

means of increasing the amounts of cellulose fermented. The investigation described in this report confirms the necessity of removing inhibiting end products for more complete fermentation and points to the use of ion exchange resins as a possible means of end-product removal.

Materials and Methods

All rumen fluid used as medium and inoculum was obtained from freshly killed animals. The paunch was opened about 10 min. after the animal was slaughtered and the contents squeezed through several layers of cheesecloth into a screw capped bottle. The bottle and contents were removed to the laboratory and fermentations were begun as quickly as possible.

Whatman No. 2 filter paper, ground finely in a Wiley mill, was used as substrate throughout the work. No attempt has as yet been made to use wood as substrate.

Lower fatty acids, formed by fermentation, were determined by the method of Neish (4). The mixture of acids (butyric, propionic, acetic, and formic) obtained was separated on a celite column and each acid was eluted and then titrated individually.

Total carbohydrate (cellulose and all carbohydrate present) was determined by the following method: a 10 ml. aliquot was withdrawn from a well-mixed test sample and added to a 50 ml. volumetric flask suspended in a cold water bath. Seventy per cent (by volume) sulphuric acid was added to the flask slowly with mixing to prevent charring, until the flask had been made up to volume. The resulting solution was left standing for one-half hour before analysis. One milliliter of this solution was then diluted to 100 or 200 ml. with distilled water to obtain a final solution containing between 10 and 50 γ glucose per ml. Five milliliters of this solution was then added to 10 ml. of fresh anthrone reagent (0.2% recrystallized anthrone in concentrated sulphuric acid) in a 22 $\times$ 175 mm. test tube suspended in a cold water bath. The reagent and the test solution were well mixed while still in the water bath. The tube was removed and placed in a boiling water bath for exactly 10 min., cooled to room temperature in a cold bath, and the blue color read in a Nefluoro-Photometer using a 525 mμ filter. Known glucose solutions were run along with each analysis in order to obtain a standard reference curve.

Experimental

Much experimentation was undertaken before the fermentor and method of procedure described hereafter were accomplished. Detailed descriptions of the preliminary experiments and procedures are omitted for the sake of clarity and brevity. A diagram of the fermentor in its present form is given in Fig. 1.

In this fermentor, provision was made for automatic control of pH. A Beckman automatic titrator, model K, was adapted to the purpose. A 2 liter Corning 6947 kettle was used as fermentor. The kettle was fitted with a lucite lid, cut from one-quarter inch stock material. The lid was clamped tightly to the kettle using a rubber gasket and the kettle clamps provided.

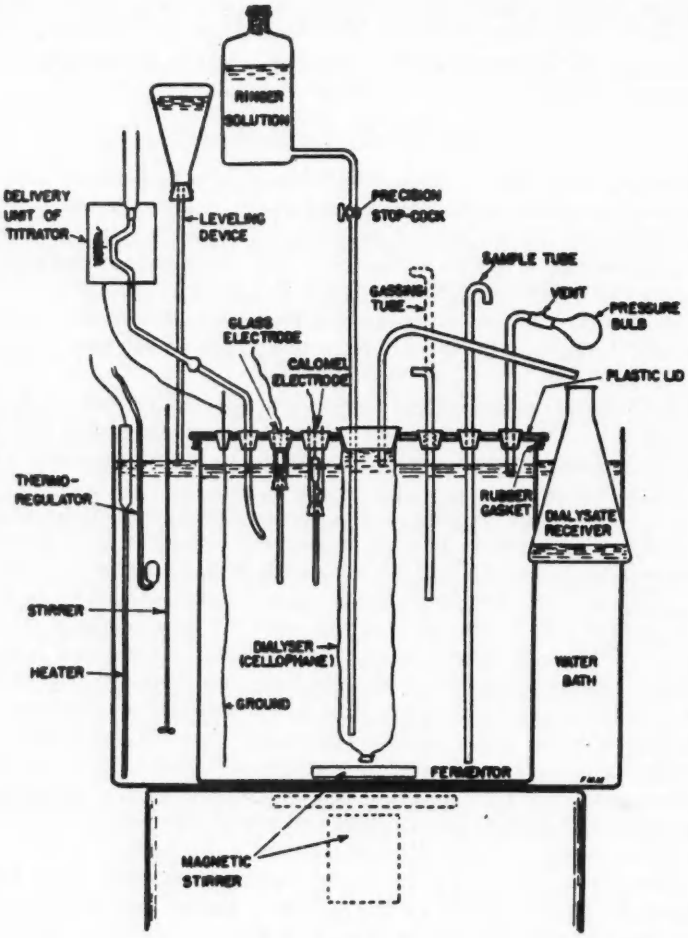

FIG. 1

The delivery unit of the titrator was set up over the fermentor. The fermentor was placed into a 12 in. × 12 in. constant temperature water bath (40° C.) with levelling device attached, and the whole assembly was mounted on top of a large Mag-mix magnetic stirrer.

To facilitate use of the titrator as a pH controller, the burette tip, ground wire, and electrodes were removed from the delivery unit of the titrator, were introduced through the lid of the fermentor, and were held in place with appropriate rubber stoppers. It was necessary to extend the ground wire and glass delivery tip. A copper wire with alligator clip attached was used

for the former and 2 mm. I.D. thick-walled tubing fitted with a 12/5 ball-and-socket joint was used for the latter. A 100 ml. burette was used in place of the burette supplied with the titrator to act as a reservoir for base. A gassing tube, sample tube, pressure bulb, and vent were installed as shown in Fig. 1. A dialysis system was provided in order that end products of fermentation could be removed. A large hole was made in the center of the lid to take a No. 10 rubber stopper with three inch dialyzing tubing attached: the latter was hanging freely in the center of the fermentor. Ringer phosphate solution with 0.2% sodium thioglycolate added was placed in the dialyzing sac, as the medium against which the end products were dialyzed. Several means were tried in an attempt to maintain optimum conditions for dialysis inside the dialyzer. One is shown in Fig. 1 in which case the contents of the sac were flushed continuously with fresh dialyzing solution using a gravity feed arrangement as shown. However, in the latest run attempted the flushing system was removed and Amberlite IRA-400 (anion) resin was placed inside the sac to absorb end products of fermentation as they passed across the membrane. The resin was kept in motion by bubbling oxygen-free nitrogen slowly into the sac.

A simple procedure was adopted for filling and running the fermentor. Ground cellulose was placed in the bottom and wetted with a solution of mineral salts. The lid with dialyzer and electrodes, etc., installed was clamped in position and the fermentor gassed free of air with oxygen-free carbon dioxide. Fresh rumen fluid was then added and the gassing continued until the charging was completed. The mixer was started and adjusted so as to just keep the cellulose in suspension and the whole mass was left to equilibrate for 15 min. A small sample was then removed from the fermentor and the pH read in a pH meter. The reading obtained was used to adjust the titrator. Saturated sodium bicarbonate solution was used to bring the pH to the desired level, the zero hour sample for analysis was then taken, and the whole apparatus then left to run automatically. A slight positive pressure of oxygen-free carbon dioxide was maintained in the fermentor at all times to displace any air that might have crept in.

To check the accuracy of the titrator as a pH controller, blank runs were tried using standard buffer solutions in the fermentor. The controls were set to hold at the pH of the buffer and the apparatus left to run 72 hr. The only drift noticeable in the instrument was a change of approximately 0.1 of a pH unit. The instrument was, therefore, considered quite satisfactory for the proposed study.

Results and Discussion

A number of runs were carried out using the above apparatus as well as modifications of it. The conditions of fermentation for the most efficient run were as follows: ion exchange (Amberlite) resin as mentioned earlier was placed inside the dialyzing sac and was replaced after each 24 hr. fermentation

period with fresh regenerated resin. Ground cellulose at an initial concentration of 4.12% was suspended and peptone as a source of nitrogen was added (approximately 0.5%). The titrator was adjusted to maintain the pH at 6.5. Saturated sodium bicarbonate solution was added through the titrator to neutralize the acids formed by fermentation. Stirring of the cellulose suspension was regulated so that the cellulose was just set in motion; however, when samples were taken for analysis, the stirring rate was increased to provide a representative sample. The results of the run are shown in Table I. The rate at which carbohydrate was decomposed in the experiment is shown best in Fig. 2 (curve *A*). After only 24 hr. of fermentation, approximately half of the cellulose originally present was consumed. In the next 24 hr., half of the substrate remaining was consumed. After 72 hr., 83% of the original 4.12% cellulose suspension had been decomposed.

For comparison, results obtained under other conditions of fermentation are also shown in Fig. 2. Curve *B* and Table II show the lower rate of decom-

TABLE I

FERMENTATION OF CELLULOSE USING ION EXCHANGE AND DIALYSIS TO REMOVE END PRODUCTS

Incubated, hr.	Carbohydrate, gm./100 ml. mash		Carbohydrate consumed, %	Total acid in fermentor,* gm./100 ml. mash
	Present	Consumed		
0	4.12	0	0	0.42
24	1.94	2.18	52.9	0.58
48	0.85	3.27	79.4	1.35
72	0.69	3.43	83.3	1.53

* *Not taking into account acid removed by dialysis and ion exchange.*

TABLE II

FERMENTATION OF CELLULOSE, END PRODUCTS REMOVED BY DIALYSIS (BATCH)

Incubated, hr.	Carbohydrate, gm./100 ml. mash		Carbohydrate consumed, %	Total acid in fermentor,* gm./100 ml. mash
	Present	Consumed		
0	2.96	0	0	0.34
24	1.83	1.13	38.2	0.74
40	1.15	1.81	61.2	0.99
64	0.43	2.53	85.6	1.21

* *Not taking into account acid removed by dialysis.*

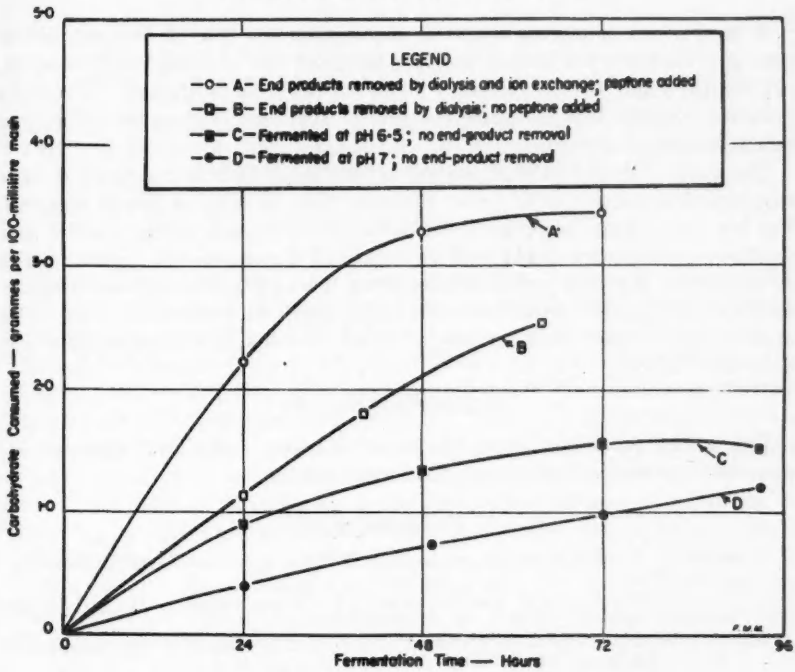

FIG. 2. Influence of different conditions on the rate of cellulose decomposition.

position obtained when no nitrogen (peptone) was added to the fermentation. Acids formed by fermentation were removed by dialysis. The content of the dialyzer was removed every 24 hr. and fresh solution added. No ion exchange resin was placed in the dialyzer as for curve *A*. Curve *A* confirmed the results obtained in a preliminary *in vitro* experiment, not reported, where the addition of peptone had markedly stimulated cellulose decomposition. The same stimulatory effect was not observed when peptone was added to test-tube cultures of the rumen organisms where no end-product removal system was present. The use of other sources of nitrogen has not been investigated as yet.

Curves *A* and *B* (Fig. 2) when compared with curves *C* and *D* show convincingly the beneficial effect of end-product removal. Under conditions of curves *C* and *D* no dialysis system was present and the rate of carbohydrate consumption was much less. It is conceivable that ion exchange without dialysis could be effective, provided fouling of the resin can be avoided. An additional advantage of this would be the possibility of obtaining the products of fermentation at high concentrations. The increase in acid concentration between the start and the end of fermentations, as shown in Tables I and II, indicates that the systems used for end-product removal were not fully efficient. If the acid concentration present in the living animal (0.3–0.4%) could be maintained, further improvements might be possible.

A comparison of curves *C* and *D* shows that the rate of fermentation is greater at the lower pH level (6.5). At a sufficient rate of end-product removal, pH control might not be necessary at all for rapid fermentation. The data presented suggest that complete removal of acid ions is more desirable than neutralization of the ions.

The study reported is of a preliminary nature. The data presented show that rapid decomposition of heavy concentrations of cellulose can be attained. Further work using improved methods of removing end products and better nutritional conditions might lead to improved fermentations. Some studies to determine the nutritional requirements, culture and subculture characteristics, and requirements for stock culture will be reported shortly. An investigation is also being carried out in which wood is used as substrate in place of cellulose.

Acknowledgments

The author is very grateful to Canada Packers Ltd., Hull, Quebec, for permission to obtain fresh rumen fluid when needed.

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NOTE

APPLICATION OF FILTER PAPER CHROMATOGRAPHY TO THE ANALYSIS OF STREPTOMYCETE PHAGE PREPARATIONS

By D. Perlman

Several years ago we found preparations of *Streptomyces griseus* phage type I (2) which formed different size plaques when mixed with the sensitive host cells in semisolid media. Study of these phage preparations using the electron microscope showed only slight differences in the morphology of the different phage particles. The length of the "tails" was rather uniform, but the size of the "heads" varied somewhat. While it was possible to separate these phage particles by isolation and propagation of the individual plaques, it was of interest to determine whether they could be separated by filter paper chromatography. Subsequent to the completion of these experiments, the paper by Shepard (3) describing the separation of the seven coli phages chromatographically in paper strips by a solvent system containing 0.1% bovine plasma albumin and 0.1 *M* sodium chloride came to our attention. Our studies have shown that other systems may be used, and suggest that Shepard was perhaps observing displacement as well as chromatography in his experiments.

In preliminary experiments it was found that many solvents commonly used in filter paper chromatography of enzymes (1) had little effect on the viability of the *S. griseus* phage. Aqueous systems containing acetone, ethanol, methanol, cellosolve, acetic acid, ammonium sulphate, and ammonium citrate had no effect on phage viability. Mixture of phage with nonaqueous solvents resulted in inactivation.

Preparations of phage type I separated into two entities when 50% aqueous acetone or methanol was used as the solvent in filter paper chromatograms. The areas of the paper containing the phage were detected by placing the chromatograms for a short time on agar inoculated with host cells. Cleared areas were found on the agar plates as a result of the presence of the phage. Efforts were then directed to carrying out these studies on a quantitative basis. Only a small percentage of the phage particles placed on the paper could be eluted by placing the paper on the agar. At first it was thought that the phage had been inactivated during air-drying of the paper. This was shown to be an erroneous hypothesis as maceration of the paper in water or dilute agar resulted in recovery of some of the phage. A number of solutions were then tested as eluants. The most effective eluants were solutions of peptone, casein, gelatine, yeast autolyzate, albumin, and zein which eluted from 50 to 100% of the phage added to the paper. As solutions of inorganic salts, carbohydrates, or amino acids were ineffective in eluting the phage from

the filter paper, it appeared that the proteins acted by displacing the phage from the paper fibers rather than by neutralizing ionic charges.

A series of experiments showed a number of solvent mixtures were suitable for the development of chromatograms. These included acetone–water in proportions of 1 : 9 to 9 : 1, methanol–water in the same proportions, and ethanol–water in proportions of 1 : 1 to 1 : 3. Whatman No. 1 filter paper was used throughout these experiments. At the end of the development period, the filter paper strip was cut into sections and each segment was eluted with 5 ml. of a 1% peptone solution. The eluate was added to Petri dishes containing *S. griseus* spores and 0.5 ml. of 1 *M* calcium chloride. Melted agar was then added and the suspension well mixed. The plates were incubated at 29° C. for 24 hr. and then the plaques were counted. Recovery of phage placed on the chromatogram ranged from 65 to 100%. When the solvent mixtures mentioned above were used to develop the chromatograms two entities were obtained, one with an R_f of 0.65–0.85 and the other with an R_f of 0.15–0.20. Repeated chromatography of each phage fraction did not result in further fractionation which leads to the conclusion that the entities were not artifacts resulting from the analytical system. The phage plaques at R_f 0.8 were of the "small" size while those at R_f 0.2 were somewhat larger.

Strains of *S. griseus* resistant to the phage forming large plaques were found to be somewhat resistant to the fraction forming small plaques. On the other hand, the strains resistant to the small-plaque forming phage were resistant to the large-plaque forming phage. Repeated propagation of the large plaque on the host (using single plaque transfers) was found to result in a mixture of plaques containing a substantial number, *ca.* 20% of the total particles, of small-plaque forming phage. Similar experiments showed that the small-plaque phage did not give rise to a mixture of particles forming large and small plaques.

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